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Aurora kinase inhibitors: which role in the treatment of chronic myelogenous leukemia patients resistant to imatinib?

Giovanni Martinelli,1 Cristina Papayannidis,1 Ilaria Iacobucci,1 Simona Soverini,1 Daniela Cillonii,2 Michele Baccarani1
1Institute of Hematology and Medical Oncology “L. and A. Seràgnoli”, University of Bologna, Italy
2Division of Hematology and Internal Medicine, Department of Clinical and Biological Science, University of Turin, Italy

Abstract

At present, there are no compounds in clinical development in the field of chronic myeloid leukemia (CML) or Philadelphia-positive (Ph+) acute lymphoblastic leukemia (ALL) that have been documented to harbor significant activity against the imatinib-resistant T315I mutation. Recent reports on the preclinical activity of some emerging tyrosine kinase inhibitors such as ON012380, VX-680 and PHA-739358 promise possible clinical efficacy against this specific Bcr-Abl mutant form. Here, we focus on the role of aurora kinase inhibitors VX-680 and PHA-739358 in blocking the leukemogenic pathways driven by wild-type and T315I-Bcr-Abl in CML or Ph+ ALL by reviewing recent research evidence. We also discuss the possibility of employing aurora kinase inhibitors as a promising new therapeutic approach in the treatment of CML and Ph+ ALL patients resistant to first and second generation TK inhibitors.

Introduction

The molecular signature of chronic myeloid leukemia (CML) and Philadelphia-positive (Ph+) acute lymphoblastic leukemia (ALL) is the Bcr-Abl hybrid gene, originating from a reciprocal t(9;22) chromosomal translocation on the 22q- derivative, commonly referred to as the Philadelphia chromosome.1 The resulting fusion protein, Bcr-Abl, displays deregulated tyrosine kinase activity and drives CML.2 The disease begins with an indolent chronic phase (CP) marked by the expansion of myeloid cells with normal differentiation, and then inexorably proceeds to advanced phases, i.e., accelerated phase (AP) and the terminal blastic phase (BP). Imatinib (Gleevec, Glivec; STI571), a relatively selective tyrosine kinase inhibitor that blocks the catalytic activity of Bcr-Abl, is now the first-line treatment for all newly diagnosed CML patients. Despite excellent clinical results, there is still a need to improve therapy for patients with CML and Ph+ ALL. More than 80% of newly diagnosed CML patients treated with imatinib in CP achieve a complete cytogenetic remission, as typified by the absence of the Philadelphia chromosome at the examination of 20 bone marrow meta-phases.3 However, residual Bcr-Abl transcripts persist in the majority of these patients, as assessed by sensitive assays such as nested reverse transcription-polymerase chain reaction, and represent the potential pool from which disease recurrence may originate. While responses in CML in CP patients have been shown to last more than five years,4 most responding patients with AP- and BP-CML, as well as those with Ph+ ALL, relapse early despite continued therapy. Resistance to imatinib is most commonly mediated by Abl kinase domain mutations.5 We and other authors have reported that approximately half CML patients have evidence of point mutations within the Abl kinase domain at the time of resistance to imatinib. Mutations target critical contact points between imatinib and Bcr-Abl or, more often, induce a conformation to which imatinib is unable to bind.1 In the remaining patients, the reasons for imatinib resistance have to be traced to Bcr-Abl gene amplification or overexpression, clonal cytogenetic evolution, or altered levels of transport molecules responsible for imatinib influx and efflux (ABC transporters, hOCT1).6 Abl mutations are at present the most extensively investigated and best characterized mechanism of resistance to imatinib. So far, at least 90 different point mutations have been isolated from relapsed CML patients who are resistant to imatinib.7,8 The clinical and pathogenetic impact of mutations varies according to their different degree of residual sensitivity to imatinib. Indeed, while certain Bcr-Abl mutations retain in vitro sensitivity to imatinib at physiologically relevant concentrations and therefore may not be clinically meaningful, others require increased doses of imatinib, and some confer a highly resistant phenotype (Table 1).9

The T315I mutation is highly resistant to imatinib

An amino acid substitution occurring at the so-called “gatekeeper” residue, i.e., threonine 315, has attracted particular interest since it confers a high level of resistance not only to imatinib therapy but also to all of the newly developed tyrosine kinase inhibitors entered in clinical trials. Co-crystal structure analysis indicates that, on binding, the hydroxyl group of threonine 315 forms a crucial hydrogen bond with imatinib.10 Moreover, the side chain of threonine also sterically controls the binding of the inhibitor to hydrophobic regions adjacent to the ATP-binding site.11 In 10-15% of imatinib-resistant patients, especially those in more advanced phases of disease, a threonine to isoleucine amino acid substitution may be observed. The T315I abrogates imatinib binding because it disrupts the above mentioned hydrogen bond and introduces a bulkier isoleucine side chain into the gatekeeper position.12 However, this explanation is not the most up-to-date. In fact, as recently demonstrated, the T315I resistance to imatinib mainly results from the breakdown of interactions between imatinib and both E286 and M290.13 As a result, biochemical and cellular IC50 values of imatinib for the T315I-Bcr-Abl have been shown to be >6400 times higher than those of wild-type Bcr-Abl (Table 1).3 Some authors have suggested that the T315I is associated with highly aggressive disease phenotype and poor outcome if no timely therapeutic reassessment is made.14,15 However, the effects of the T315I mutation on kinase activity in vitro and transforming efficiency of Bcr-Abl in vitro and in vivo have been very recently investigated, suggesting that in the absence of imatinib, there is nei-

[Hematology Reviews 2009; 1:e1]
ther increased kinase activity nor any growth advantage for cells carrying T315I-Bcr-Abl as compared to wild-type Bcr-Abl.5

The two second-generation inhibitors in clinical development, dasatinib and nilotinib, are ineffective against the T315I mutant

To counteract the problem of resistance due to point mutations, several second-generation inhibitors have been synthesized and tested in pre-clinical assays: nilotinib (AMN107),8,16-18 dasatinib (BMS-354825),9,15-21 bosutinib,21 VX-680,21 AP23464,21,27 PD166326, PD180970 and PD173955,18,19,32 and ON012380.33 Two of them are currently being evaluated in phase II clinical trials – the dual-specificity Src/Abl inhibitor dasatinib and the imatinib derivative nilotinib. Dasatinib is a novel, dual Src and Abl inhibitor entered in clinical trials. It has been shown to be ~300 times more potent than imatinib in Bcr-Abl inhibition assays. Excellent results in terms of hematologic and cytogenetic response in CML and Ph+ ALL patients resistant to imatinib have been reported after dasatinib administration.34 Pre-clinical studies have demonstrated that dasatinib is active against at least fourteen imatinib-resistant Bcr-Abl mutants (M244V, Q252E, Q252H/R, Y253F/H, E255K, E255V, F317L, M351T, E355G, F359V, H396R, F486S).

The only imatinib-resistant Bcr-Abl isoform that was clearly insensitive to dasatinib was the T315I mutant, which retained kinase activity even in the presence of micromolar concentrations of the compound (Table 1).19 Accordingly, imatinib-resistant patients harboring the T315I mutation have been shown not to benefit from dasatinib in the recent phase I trial.34

Nilotinib is a close relative of imatinib with more than 20-fold improved affinity for wild-type Bcr-Abl.16 It is highly efficacious in patients with imatinib-resistant Ph+ CML. In vitro experiment with cell lines transformed with mutated forms of Bcr-Abl showed IC50 proliferation inhibition for most mutations with the exception of the T315I, which remains refractory to nilotinib8 (Table 1). Accordingly, clinical responses have been observed in patients with various imatinib-resistant Bcr-Abl mutations but not in patients positive for the T315I in the recent phase I trial.35

Despite the pressing need for a clinically effective T315I-Bcr-Abl inhibitor, relatively few pre-clinical candidates have been reported. A potential pitfall might be the tendency to screen initially for Abl kinase inhibition rather than for T315I-specific inhibition. A promising approach is to design inhibitors targeting other regions of Bcr-Abl. For example, ON012380, a putative substrate-competitive inhibitor, exhibited low nanomolar activity against imatinib-resistant Bcr-Abl mutants, including the T315I, in biochemical and cellular assays.33

Aurora kinases as targets for cancer

Between these new promising drugs, VX-680 and PHA-T73958, two aurora kinase A, B and C inhibitors, have a leading place. The aurora kinases are a family of serine/threonine kinases involved in many cellular functions, including progression through mitosis, by regulating spindle formation, chromosome segregation and cytokinesis.35-37 The overexpression of aurora kinases has been reported in many human solid tumors, leading to defects in centrosome function, aberrant spindle assembly, misalignment of chromosomes, abnormal cytokinesis and genetic instability, determining the activation of oncogenic pathways.35-37 Many authors reported an aberrant expression of the aurora A and B kinases also in leukemia cells, suggesting a potential role of these molecular targets in the treatment of CML and ALL.40-41 Aurora kinase function is mediated by the phosphorylation of several substrates that have important roles in cell division, such as proteins survivin, CENP-A and serine 10 on histone H3.37 The aurora kinases range in size from 309 to 403 amino acids. They have a C terminal domain that is

<table>
<thead>
<tr>
<th>Abl variant</th>
<th>Imatinib IC50 (nM)</th>
<th>Fold-change</th>
<th>Cellular proliferation Nilotinib IC50 (nM)</th>
<th>Fold-change</th>
<th>Dasatinib IC50 (nM)</th>
<th>Fold-change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>260</td>
<td>1</td>
<td>13</td>
<td>1</td>
<td>0.8</td>
<td>1</td>
</tr>
<tr>
<td>M244V</td>
<td>2,000</td>
<td>8</td>
<td>38</td>
<td>3</td>
<td>1.3</td>
<td>2</td>
</tr>
<tr>
<td>Q250E</td>
<td>1,350</td>
<td>5</td>
<td>48</td>
<td>4</td>
<td>1.8</td>
<td>2</td>
</tr>
<tr>
<td>Q252H</td>
<td>1,285</td>
<td>5</td>
<td>70</td>
<td>5</td>
<td>3.4</td>
<td>4</td>
</tr>
<tr>
<td>Y253F</td>
<td>3,475</td>
<td>13</td>
<td>125</td>
<td>10</td>
<td>1.4</td>
<td>2</td>
</tr>
<tr>
<td>Y253H</td>
<td>&gt;6,400</td>
<td>&gt;25</td>
<td>450</td>
<td>35</td>
<td>1.3</td>
<td>2</td>
</tr>
<tr>
<td>E255K</td>
<td>5,200</td>
<td>20</td>
<td>200</td>
<td>15</td>
<td>5.6</td>
<td>7</td>
</tr>
<tr>
<td>E255V</td>
<td>&gt;6,400</td>
<td>&gt;25</td>
<td>430</td>
<td>33</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>F311L</td>
<td>480</td>
<td>2</td>
<td>23</td>
<td>2</td>
<td>1.3</td>
<td>2</td>
</tr>
<tr>
<td>T315I</td>
<td>&gt;6,400</td>
<td>&gt;25</td>
<td>&gt;2000</td>
<td>&gt;154</td>
<td>&gt;200</td>
<td>&gt;250</td>
</tr>
<tr>
<td>F317L</td>
<td>1,050</td>
<td>4</td>
<td>50</td>
<td>4</td>
<td>7.4</td>
<td>9</td>
</tr>
<tr>
<td>M351T</td>
<td>880</td>
<td>3</td>
<td>15</td>
<td>1.2</td>
<td>1.1</td>
<td>1.4</td>
</tr>
<tr>
<td>F359V</td>
<td>1,825</td>
<td>7</td>
<td>175</td>
<td>13</td>
<td>2.2</td>
<td>3</td>
</tr>
<tr>
<td>L387M</td>
<td>1,000</td>
<td>4</td>
<td>49</td>
<td>4</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>H396P</td>
<td>850</td>
<td>3</td>
<td>41</td>
<td>3</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>H396R</td>
<td>1,750</td>
<td>7</td>
<td>41</td>
<td>3</td>
<td>1.3</td>
<td>2</td>
</tr>
</tbody>
</table>
responsible for regulation of the protein levels via proteasomal degradation; a highly conserved catalytic domain; and a short N-terminal domain that varies in length between the kinases and contributes to the differing locations of the kinases within cells.43 (Figure 1).

The aurora A isotype (also known as aurora, Aurora-2, AIK, AIR-1, AIRK1, AYK1, BTAK, Eg2, MnlAIK1 and STK15) is widely expressed in proliferating normal tissues, with expression being cell-cycle-dependent and peaking at the G2/M point of the cell cycle. During mitosis, the kinase is virtually confined to the spindle poles, where it is needed for centrosome separation and maturation.44 An overexpression of aurora A causes an increase in centrosome numbers and aneuploidy, leading to the transformation of mammalian cells and also causes resistance to apoptosis induced by taxol in human cancer cell lines. Moreover, this kinase is a key regulatory component of the p53 pathway as its overexpression leads to increased p53 degradation, which facilitates oncogenic transformation.46 Human aurora A has been proposed as a "druggable" target in several tumors including pancreatic,46,47 hepatocellular,48 breast,49 nonendometriod,50 and ovarian carcinomas,51 gliomas52 and aggressive non-Hodgkin’s lymphoma.53 Aurora B (also known as Aurora-1, AIM-1, AIK2, AIR-2, ARK2, IAL-1 and STK12) activity is required for bipolar chromosome orientation and condensation.54 Aurora B kinases are chromosomal passenger proteins, which are found in cells in a complex with inner centromere protein (INCENP) and survivin. The overexpression of an aurora B kinase-dead mutant (K-R) causes multiple defects in the mitotic machinery, including the loss of kinetochore attachment to microtubules and the exit from mitosis without anaphase or cytokinesis.55 Increased Aurora B expression correlates with increased grade in glioma and colon cancer and with anaplasia in thyroid carcinoma.56 Aurora C (also known as AIK3) expression plays a role in spermatogenesis at the time when cells assemble the two meiotic spindles and also cooperates with aurora B to regulate mitotic chromosome dynamics in mammalian cells.57 Aurora C overexpression has been detected in tumor cell lines in vitro and in biopsy samples from colorectal carcinoma.58

**Novel aurora kinase inhibitors effective against the T315I-Bcr-Abl**

Several compounds have been pre-clinically screened for their inhibitory activity against aurora kinases (VX680, MLN8054, AZD1152, R766, R763, PHA-739358, AT9283) and many of

**Table 2. Novel compound aurora-kinase inhibitors in clinical trial development.**

<table>
<thead>
<tr>
<th>TK inhibitor</th>
<th>Company</th>
<th>Phase</th>
<th>Target(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MK-0457</td>
<td>Merck</td>
<td>I</td>
<td>Aurora, FLT-3, JAK-2</td>
</tr>
<tr>
<td>PHA-739358</td>
<td>Nerviano</td>
<td>II</td>
<td>Aurora</td>
</tr>
<tr>
<td>KW-2449</td>
<td>Kyowa</td>
<td>I</td>
<td>Aurora</td>
</tr>
<tr>
<td>Deciphera</td>
<td>Deciphera</td>
<td>I</td>
<td>Abl</td>
</tr>
<tr>
<td>A5703569</td>
<td>Merck Serono</td>
<td>I-II</td>
<td>Aurora, Abl, JAK-2</td>
</tr>
<tr>
<td>AZD1152</td>
<td>Astra-Zeneca</td>
<td>I-II</td>
<td>Aurora</td>
</tr>
</tbody>
</table>

**Table 3. Comparison between the binding affinity of imatinib and of the aurora kinase inhibitors BIRB-796 and MK-0457 for wild-type and drug-resistant Abl variants.** Adapted from [21].

<table>
<thead>
<tr>
<th>Abl variant</th>
<th>Imatinib</th>
<th>BIRB-796</th>
<th>MK-0457</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.230</td>
<td>0.610</td>
<td>0.100</td>
</tr>
<tr>
<td>Q252H</td>
<td>0.021</td>
<td>0.014</td>
<td>0.005</td>
</tr>
<tr>
<td>Y253F</td>
<td>0.083</td>
<td>0.205</td>
<td>0.085</td>
</tr>
<tr>
<td>E255K</td>
<td>0.230</td>
<td>0.610</td>
<td>&gt;20,000</td>
</tr>
<tr>
<td>M351T</td>
<td>0.021</td>
<td>0.014</td>
<td>0.005</td>
</tr>
<tr>
<td>T315I</td>
<td>0.083</td>
<td>0.205</td>
<td>0.085</td>
</tr>
<tr>
<td>H396P</td>
<td>0.230</td>
<td>0.610</td>
<td>&gt;20,000</td>
</tr>
</tbody>
</table>

**Table 4. Comparison between the binding affinity of imatinib and of the aurora kinase inhibitors PHA-739358 and MK-0457 for wild-type and drug-resistant Abl variants.** Adapted from [63].

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>E255V (P loop)</th>
<th>T315I</th>
<th>M351T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imatinib</td>
<td>0.230</td>
<td>0.610</td>
<td>&gt;20,000</td>
<td>0.100</td>
</tr>
<tr>
<td>PHA-739358</td>
<td>0.021</td>
<td>0.014</td>
<td>0.005</td>
<td>0.015</td>
</tr>
<tr>
<td>MK-0457</td>
<td>0.083</td>
<td>0.205</td>
<td>0.085</td>
<td>0.045</td>
</tr>
</tbody>
</table>

[Hematology Reviews 2009; 1:e1]
them are being tested in clinical phase I/II trials (Table 2). MK-0457 (VX-680) is a pan-aurora kinase inhibitor with demonstrated in vitro activity against wild-type and mutated Bcr-Abl, including the T315I form, as well as FLT3 and JAK-2.2 Fascinatingly, Carter et al. have found that the aurora kinase inhibitor VX-680, already in phase I trials, and the p38 inhibitor BIRB-796, in clinical trials for inflammatory disease, inhibit the imatinib- and dasatinib-resistant T315I-Bcr-Abl with high affinity (Tables 3 and 4). In fact, contrasting results related to this compound have been published. In particular, BIRB-796 binds with good affinity to T315I-Bcr-Abl (Kd = 40 nM), but has significantly weaker affinity for wild-type and other imatinib-resistant forms of Abl, with Kd values >1 μM.2 In contrast, as reported by other authors, the compound fails to inhibit the proliferation of cells expressing T315I, suggesting a lack of clinical benefit for patients harboring such a mutation.22

In a recent phase I-II study, MK-0457 was shown to be active in patients with T315I phenotype-refractory CML or Ph-positive ALL, with no significant extramedullary toxicity.62 Because of a potential heart safety issue revealed in one patient who experienced QTc prolongation, the enrollment on phase II protocol was halted in November 2007. Furthermore, an innovative phase I clinical study of sequential and concomitant treatment with dasatinib and MK-0457 has been conducted, based on the suggestion that such a combinatorial approach would suppress the emergence of T315I and other resistant clones, improving upon the response rate for dasatinib and the durability of response. To date, 3 patients with wild-type chronic myeloid leukemia (CML) or Ph-positive acute lymphoblastic leukemia (ALL) have been enrolled, and this innovative therapeutic combination showed a relevant hematologic activity and a good safety profile. PHA-739358 is a small molecule that selectively inhibits the ATP site of Aurora-A (IC50=13 nM) and Aurora-B (IC50=79 nM).63 Starting from the rationale that aurora kinases play an important role in mitosis and that the interruption of their function has significant potential in the treatment of cancer, the drug, formulated for intravenous infusion, is being developed for therapeutic use in solid tumors and in patients with Philadelphia positive leukemias. Interestingly, PHA-739358, when tested against a panel of more than 30 kinases, has shown a strong cross-reactivity with c-Abl (IC50=25 nM). Its inhibitory activity on ABL in cells was confirmed in K562 leukemia cells which bear the Philadelphia chromosome related translocation Bcr-Abl. Furthermore PHA-739358 inhibits phosphorylation of Tyr412, which is located in the kinase activation loop of Abl and is also active against the T315I mutant of Abl, which is resistant to other ATP competitive inhibitors in the clinic, such as gleevec, and second generation TK inhibitors. A multicentric phase I/I study, aimed to test PHA-739358 in patients with chronic, accelerated or blast phase CML relapsing on gleevec or c-Abl therapy and preferably with T315I mutation in Bcr-Abl kinase is ongoing.

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Binding mode of VX-680 and PHA-739358 to Abl

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ARM (Figure 2). A recent study showed that VX-680 forms a hydrogen bond with the strictly conserved Asp381 of the Asp-Phe-Gly (DFG) motif in the Abl kinase domain and maintains it in an orientation close to one that is normally seen in active kinases, although the activation loop of Abl is not phosphorylated in this structure (Figure 3). Furthermore, VX-680 does not deeply penetrate into the kinase domain as imatinib does and it is anchored to it by four hydrogen bonds. Three of these are formed between two carbonyl groups (Glu316 and Met318) and an amide nitrogen (Met318) in the “hinge region” of the kinase and three nitrogen atoms, one in the linker between the pyrimidine group and the methylpyrazole group, and the other two in the methylpyrazole group. These bonds are a common feature of kinase inhibitors and are independent of the sequence of the kinase.

Likewise, the fourth hydrogen bond, made by VX-680 to the side chain of Asp381 of the DFG motif, is to a strictly invariant catalytic residue. Using these four anchors, the inhibitor makes contact with 14 side chains within the kinase domain, eight of which are identical between Abl and aurora. One of the non-conservative substitutions is at the gatekeeper position, where Thr315 in Bcr-Abl is replaced by Leu210 in aurora A kinase (Figure 4). The side chains of isoleucine (at position 315 of Bcr-Abl) and leucine (at position 210 of aurora A kinase) can be accommodated readily between the two sides of the “Y” of VX-680. For this reason, VX-680, in contrast to imatinib, is able to inhibit the kinase activity of both wild-type Bcr-Abl and T315I-Bcr-Abl. To understand the structural basis of the capability of PHA-739358 to bind and inhibit the T315I mutant, the crystal structure of the inhibitor-protein complex was determined (Figure 5). The protein is in the typical conformation of active kinases, with the activation loop in the extended DFG “in” conformation. Indeed, Asp381 points into the active site and interacts with Mg2+ ion that occupies a position similar to the one usually seen in the structures of kinases in complex with ATP. The glycine loop adopts an extended conformation, in contrast to the other publicly available Abl structures where the loop is more distorted, which could be due to the specific binding mode of our inhibitor. The purified T315I Abl kinase domain used for crystallization experiments is predominantly phosphorylated on the activation loop at Tyr393, whereas Tyr253, Tyr257, and Tyr264 are phosphorylated at lower levels. These interactions probably stabilize the active conformation of the activation loop, which is, however, very similar to the structures reported for dasatinib in complex with the WT Abl kinase domain and of MK-0457 in complex with the Abl mutant H396P.4 The mutation of the threonine to the more bulky isoleucine does not seem to cause any widespread conformational changes but creates a steric hindrance that would interfere with the binding of inhibitors, such as imatinib, nilotinib, and dasatinib, which make use of the hydrophobic pocket. The binding mode of PHA-739358 is very similar to that reported for the complex of the same compound with aurora A (Figure 5B and D), although the conformation of the proteins around the ATP-binding site shows some differences because in the aurora A structure the DFG motif is more similar to the “out” conformation. However, all of the essential contacts between PHA-739358 and Abl T315I involve highly conserved elements. The molecule makes three hydrogen bonds with the protein...
Figure 6. Comparison of PHA-739358 complex with imatinib and MK-0457 complexes. (A) and (B) PHA-739358 complex (green carbon atoms) superimposed with the structure of imatinib (magenta carbon atoms). The gatekeeper residues Ile315 of PHA-739358 complex (A) and Thr315 of the imatinib complex (B) are shown with van der Waals spheres. In the T315I mutant, the isoleucine side chain causes a steric clash with imatinib; in addition, the hydrogen bond between imatinib and the side chain oxygen of threonine is lost. (C) Structure of Abl-PHA-739358 complex (green carbon atoms) superimposed on the structure of MK-0457. [Reprinted and adapted with permission of AACR from: Modugno M, et al. Crystal structure of the T315I Abl mutant in complex with the aurora kinases inhibitor PHA-739358. Cancer Res 2007 Sep 1;67(17):7987-90].

Conclusions

The T315I is responsible for approximately 15% of the cases of relapse in CML and Ph+ ALL patients on imatinib therapy. The clinical relevance of this mutant is likely to increase considerably as to date it seems to represent the main mechanism of resistance to dasatinib and nilotinib, the second-generation inhibitors already being developed clinically. Structural analyses indicate that the substitution of threonine with isoleucine at residue 315 eliminates a crucial hydrogen-bonding interaction and introduces a steric clash which abrogates binding and effective inhibition of Bcr-Abl by imatinib as well as by several novel inhibitors. A possible approach to the development of second-line strategies overcoming resistance induced by the T315I mutation is to design inhibitors binding regions of Bcr-Abl other than the ATP binding pocket. An intriguing alternative is to explore the possibility of whether molecules that have been developed as inhibitors for other protein kinases and are already undergoing clinical trials might include the T315I-Bcr-Abl mutant among their off-targets. Although off-target activity may lead to undesirable side effects, it has to be recognized that focusing on compounds that are already being tested in clinical practice may speed up the development of successful therapeutic strategies. Recent studies have shown that MK-0457 (VX-680) and PHA-739358, two small-molecule aurora kinase inhibitors, have in vitro activity against the T315I-Bcr-Abl. Moreover, preliminary data showed promising clinical efficacy in patients affected by Philadelphia positive leukemias, relapsing or resistant to first and second generation TK inhibitors. Such a remarkable efficacy raises the question of whether aurora kinases may also harbor some pathogenetic significance in CML and/or Ph+ ALL or may be selectively deregulated by the T315I-Bcr-Abl, and whether auroras may be a suitable secondary target for inhibition.

References

2. Groffen J, Stephenson JR, Heisterkamp N,


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Double versus single high-dose melphalan 200 mg/m² and autologous stem cell transplantation for multiple myeloma: a region-based study in 484 patients from the Nordic area

Bo Björkstrand,1 Tobias W. Klausen,2 Kari Remes,2 Astrid Gruber,3 Lene M. Knudsen,2 Olav J. Bergmann,3 Stig Lenhoff,3 Hans E. Johnsen2,4

Departments of Hematology at 1Huddinge University Hospital, Sweden, 2Copenhagen University Hospital, Herlev, Denmark 3Department of Medicine, University Central Hospital, Turku, Finland 4Karolinska Hospital, Karolinska Institute, Stockholm, Sweden 1Lund University Hospital, Lund, Sweden 2Ålborg Hospital, University of Århus Denmark for the Nordic Myeloma Study Group, Denmark

Abstract

Autologous stem cell transplantation is still considered the standard of care in young patients with multiple myeloma (MM). This disease is the most common indication for hematopoietic stem cell transplantation and much data support the benefit of this procedure. Results of randomized studies are in favor of tandem autologous transplantation although the effect on overall survival is unclear. Based on sequential registration trials in the Nordic area, we aimed to evaluate the outcome of conventional single or double HDT.

During 1994-2000 we registered a total of 484 previously untreated patients under the age of 60 years at diagnosis who on a regional basis initially were treated with single [Trial NMSG #594 and #7/98 (N=383)] or double [Trial Huddinge Karolinska Turku Herlev (N=101)] high-dose melphalan (200 mg/m²) therapy supported by autologous stem cell transplantation.

A complete or very good partial response was achieved by 40% of patients in the single transplant group and 60% of patients in the double transplant group (p=0.0006). The probability of surviving progression free for five years after the diagnosis was 25% (95% CI 18-32%) in the single transplant group and 46% (95% CI 33-55%) in the double transplant group (p=0.0014). The estimated overall five-year survival rate was 60% in the single transplant group and 64% in the double transplant (p=0.9). In a multivariate analysis of variables, including single versus double transplantation, ß2 microglobulin level, age, sex and disease stage, only ß2 microglobulin level was predictive for overall survival (p=0.0001) and progression free survival (p<0.001). In accordance with these results, a 1:1 case-control matched comparison between double and single transplantation did not identify significant differences in overall and progression free survival.

In this retrospective analysis up front double transplantation with melphalan (200 mg/m²) as compared to single transplantation did not seem to improve the final outcome among patients in the Nordic area. These data are in accordance with recent publications from the Bologna 96 trial indicating that a second transplant should not be recommended up front as standard care.

Introduction

Multiple myeloma (MM) is the second most common hematologic cancer after non-Hodgkin’s lymphoma. More than 50,000 patients in Europe alone have MM and about half of these patients are diagnosed when they are younger than 65 years of age, and increasingly detected under the age of 40.

Today, MM is the most common indication for high-dose therapy supported by hematopoietic stem cell transplantation in the world, and more data support the benefit of this procedure. These remarkable results radically altered the disease management in patients below 65 years of age. Thus, stem cell transplantation has been recommended for these patients as part of the initial therapy or at the time of disease progression. However, the median duration of response is short and almost all patients ultimately relapse.19

The InterGroupe Francophone du Myéloïde (IFM) took the next logical step and asked if the combination of two cycles of high-dose therapy and hematopoietic stem cell rescue might improve survival. The group assigned 399 patients with untreated MM below 60 years of age to receive VAD (vincristine, adriamycin and dexamethasone) as induction therapy and afterwards assigned these patients randomly to single or double transplantation conditioned by melphalan 140 mg/m² and TBI (standard single transplantation) or melphalan 140 mg/m² followed by 140 mg/m² and TBI (experimental double transplantation).19 Following a median observation of approximately six years, response rates in the two groups were not significantly different, but the probabilities of event free survival and overall survival were prolonged with a double transplant benefit that had not been evident in early analyses.19 This important study demonstrates that double transplantation is one of the options for treating myeloma, particularly those younger than 60 years of age who have a suboptimal response to a single transplant.

However, the IFM study has raised a number of questions. First and most important, do the beneficial responses in the double transplants...
plant group reflect the higher total dose of melphalan? In other words, is a single transplant with the use of maximally tolerated doses of melphalan (200 mg/m²) as effective as a double transplantation strategy with the high dose of melphalan administered twice?

Second, what should be recommended, as only one of several phase II-III studies has documented an effect on overall survival?11,12

Without doubt the IFM study has to be considered the Proof of Principle but in the light of the study design, as well as the overall results from other studies, it is still unknown if a second transplant should be recommend-
ed in all cases, even if the response to the first transplant has been inferior.

In this unclear situation we now see alternative progressive in the treatment of MM by new drugs currently being analyzed in randomized trials. In the near future, ongoing studies will clarify the role of these novel agents, including thalidomide and its analogs, and bortezomib etc., in the context of autolo-
gous stem cell transplantation. However, trial designs including consolidation therapy such as that planned by the NMSG may be hampered by a double autologous transplantation strategy, not yet documented to have an effect on survival. Here the Nordic group reports the data analysis of a total of 484 MM patients transplanted from 1994-2000 including double transplantation of 101 patients. The conclusions are based on results from two sequen-
tial phase II trials evaluating double transplantation in 4 selected centers (Huddinge, Karolinska, Turku and Herlev) by comparing the outcome with data from 383 single transplanted patients included in trial NMSG #5/94 and #7/98 from the other centers.13,14

Design and Methods

Approval and patient eligibility

The scientific protocols were reviewed and approved by the regional ethics committees in Denmark, Sweden, Finland and Norway, and all patients gave written informed consent before study entry. Patients less than 60 years of age who had Durie-Salmon stage I with at least one bone lesion, II, or III myeloma were eligible. The criteria for exclusion were prior treatment for myeloma, another cancer, abnormal cardiac function, chronic respira-

tory disease, abnormal liver function or psychi-

atric disease.

Design and aims of the program

This study was planned to include previously untreated patients under the age of 60 years at diagnosis who on a regional basis initially were treated with single [Trial NMSG #5/94 (N=383)] or double [Trial HKTH (N=101)] high-dose melphalan (200 mg/m²) therapy supported by autologous stem cell transplantation. The aim was to evaluate the outcome of conventional single or double HDT.

Double transplant study population: HKTH

From June 1994-June 2000, 101 patients with newly diagnosed myeloma <60 years were entered into a phase II trial evaluating double high-dose melphalan (200 mg/m²) therapy with autologous stem cell support. This included patients from Huddinge and Karolinska Hospitals in Stockholm, Sweden, Turku University Hospital in Finland, and from June 1997 Herlev University Hospital, Copenhagen in Denmark. This trial covered a population of 3 million. The number of new cases of myeloma <60 years in this population during the study period was estimated to be 200 patients.

Single transplant population NMSG #5/94 and NMSG #7/9814

From March 1994 until June 1997, 122 Swedish patients with newly diagnosed myeloma <60 years were entered into NMSG #5/94 trial evaluating one cycle of high-dose melphalan therapy with autologous stem cell support. One hundred and seven of these were treated according to the specified treatment protocol and received single transplantation. This trial covered a population of 15 million. A total of 348 Nordic patients were reported to the study secretariat. The expected number of new cases of myeloma <60 years in this population during the study period was estimated to be 450. In this trial, a highly significant survival advantage was found for high-dose melphalan therapy, with a prolongation of the median survival from 44 to 62 months.1

NMSG #7/98

From January 1998 until June 2000, 452 patients <65 years were registered in a similar trial evaluating high-dose melphalan with autologous stem cell support, and using a matched historical patient group as control. Of these, 276 Swedish, Norwegian and Danish patients aged <65 years were treated according to the specified treatment protocol and received single transplantation. This trial also covered a population of 15 million. A total of 452 patients were reported to the study secre-

NHSMG #5/941 and NMSG #7/9814 NMSG #5/94

From June 1994-June 2000, 101 patients with newly diagnosed myeloma <60 years were entered into phase II trial evaluating double high-dose melphalan (200 mg/m²) therapy with autologous stem cell support. This included patients from Huddinge and Karolinska Hospitals in Stockholm, Sweden, Turku University Hospital in Finland, and from June 1997 Herlev University Hospital, Copenhagen in Denmark. This trial covered a population of 3 million. The number of new cases of myeloma <60 years in this population during the study period was estimated to be 200 patients.

Criteria for response

Complete response was defined as the dis-

appearance of M-protein from serum and urine in agarose gel electrophoresis and < 5% plasma cells in a bone marrow aspirate. Very good partial remission (VGPR) was 90-99% reduc-

tion of M-protein. Partial response was defined by at least a 50% reduction of the initial serum M-protein concentration and a reduction of Bence-Jones proteinuria to <0.2 g/24 h. Minor response was defined by a 25-50% reduction of the initial serum M-protein concentration and a reduction in Bence-Jones proteinuria by at least 50% but exceeding 0.2 g/24 h.

No statistically significant differences were observed between the groups at any stage of the treatment with regard to these comparisons. To fulfill the criteria for complete, partial, or minor response, the patients were not allowed to have any other signs of myeloma progression, such as persisting hypercalcemia or progressive renal insufficiency, skeletal disease, or bone marrow insufficiency due to plasma cell infiltration. Progression was defined by a confirmed increase in the serum M-protein concentration by more than 25% from the level at the time of best response, an increase of Bence-Jones proteinuria to more than 1.0 g/24 h, or other unequivocal signs of disease progression, such as hypercalcemia, progressive skeletal disease, or soft-tissue plasmacytoma. Progression, death without progression, and occurrence of a secondary malignancy were all considered as events. Event free and overall survival was calculated from the start of therapy.
Follow-up evaluation
All registered patients were followed until death. Patients were evaluated before the start of phase II and phase III, and thereafter every sixth week.

Statistical analysis
All analyses were performed on treatment received basis and are not an intention-to-treat study. The proportions of patients with a given characteristic were compared using Fisher’s exact test for variables with frequency scale and Wilcoxon rank-sum test for the remaining variables. Event free and overall survival rates were calculated according to the Kaplan-Meier method, and survival comparisons between groups were made by the log-rank test. The Cox proportional hazards regression model was used to estimate the prognostic importance of different variables. Age, bone marrow plasma cells, blood hemoglobin, serum calcium, serum creatinine, blood platelets, and serum albumin were included as continuous variables. The following variables were dichotomized: sex (male vs. female), stage according to Durie and Salmon (I or II vs. III), M-protein class (IgG vs. other; IgA vs. other; light chains only vs. other), and osteolytic bone lesions (none vs. limited or advanced). In the multivariate analyses, forward stepwise variable selection was used.

Results

Baseline characteristics at diagnosis
Table 1 shows the base-line clinical and demographic characteristics of the 484 patients entering this analysis. There were no differences between the treatment groups but for disease stage.

Response rates in the collaborative trials
Following the final preparative treatment with high-dose melphalan and autologous stem cell transplantation, the overall rates of complete or very good partial response for patients who actually received a single or a double transplant (Table 2) were in the collaborative trials 60% and 40%, respectively (p=0.0006).

The survival outcome
In the single transplant group, the median follow-up was 34 months (range, 12-115) from the time of transplantation. The median durations of event free, progression free, and overall survival were 46, 46, and 76 months, respectively. The estimated probabilities of event-free, progression free, and overall survival five years after the inclusion were 44% (Figure 1a), 45%, and 64% (Figure 1b), respectively. Of the 33 deaths in this group, 61% were attributed to myeloma, while 3% were related to the transplantation procedure. As compared with single transplantation, double transplantation improved progression free survival (p=0.001) (Figure 1b) whereas overall survival was similar (p=0.9) (Figure 1a).

Table 1. Base-line characteristics of the patients according to treatment group.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Double transplantation</th>
<th>Single transplantation</th>
<th>p-value</th>
</tr>
</thead>
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<tr>
<td>N</td>
<td>383</td>
<td>383</td>
<td>0.4</td>
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<tr>
<td>Age*</td>
<td>55 years</td>
<td>54 years</td>
<td>0.4</td>
</tr>
<tr>
<td>Sex (female, male)</td>
<td>44/57; (44 and 56%)</td>
<td>151/232; (39 and 61%)</td>
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<tr>
<td>β2 microglobulin*</td>
<td>2.8 mg/L</td>
<td>3.4 mg/L</td>
<td>0.1*</td>
</tr>
<tr>
<td>Stage I/II/III</td>
<td>12/23/64; (12, 23 and 65%)</td>
<td>16108/259; (4, 28 and 67%)</td>
<td>0.015*</td>
</tr>
</tbody>
</table>

*Median values; *Mann-Whitney test; *Fisher’s exact test.

Table 2. Comparison between double and single transplantation.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Double transplantation</th>
<th>Single transplantation</th>
<th>p-value</th>
</tr>
</thead>
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<td>N (data/missing)</td>
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<tr>
<td>CR</td>
<td>56 (57%)</td>
<td>139 (36%)</td>
<td>0.0006*</td>
</tr>
<tr>
<td>CR + VGPR</td>
<td>59 (60%)</td>
<td>155 (40%)</td>
<td>0.0006*</td>
</tr>
<tr>
<td>OS % censored</td>
<td>See Figure 1a</td>
<td>See Figure 1a</td>
<td>0.9*</td>
</tr>
<tr>
<td>OS median (years)</td>
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<td>6.5</td>
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</tr>
<tr>
<td>PFS % censored</td>
<td>See Figure 1b</td>
<td>See Figure 1b</td>
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</tr>
<tr>
<td>PFS median (years)</td>
<td>3.8</td>
<td>2.7</td>
<td>0.9*</td>
</tr>
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</table>

*Mann-Whitney test; Fisher’s exact test; Log rank test (See Figure 1a and b)
Discussion

Double autologous transplantation regimens have been used to treat myeloma for the past decade, although the advantage over single transplantation is unclear. Despite the favorable results reported by IFM, it remains the case that progressive myeloma will develop in almost 80% of patients within seven years after they have undergone double transplantation. This unclear situation is further extended by progress in the number of new drugs currently being analyzed in randomized trials. In the near future, ongoing studies will clarify the role of inflammatory mediators and proteasome inhibitors in the context of autologous stem cell transplantation. However, trial design including consolidation therapy with some inhibitors in the context of autologous transplantation was a major concern. However, the hematopoietic reconstitution was similar after one or two transplantations but the rates of death caused by toxic effects were increased in the IFM study. Analysis of the present phase II trials after a median follow-up period of 3-4 years documented significant improved response rates and have not been included in this analysis. This number is in accordance with the literature. The most common reasons were a decision for allogeneic transplantation, poor performance status, and poor stem cell collection owing to an insufficient response after the initial VAD treatment.

The risk of life-threatening toxic effects due to double transplantation was a major concern. However, the hematopoietic reconstitution was similar after one or two transplantations but the rates of death caused by toxic effects were increased in the IFM study. Analysis of the present phase II trials after a median follow-up period of 3-4 years documented significant improved response rates and have not been included in this analysis. This number is in accordance with the literature. The most common reasons were a decision for allogeneic transplantation, poor performance status, and poor stem cell collection owing to an insufficient response after the initial VAD treatment.
when combined with dexamethasone. 16
apy-resistant disease. Thalidomide therapy is
induces responses even in patients with ther-
as a potent immunomodulatory drug that
Thalidomide, once infamous, has shown itself
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patients have become a fertile testing ground
treatment have been fast and furious. MM
survival differences, first identified 4-5 years fol-
might explain the very late significant sur-
the studies may have been too small to pro-
ive a basis for definitive conclusions. One
major concern in the IFM trial is that the
power of the study was calculated based on
evaluation of response to therapy and not on
survival estimation. Response rates analyzed
an intention-to-treat basis in the two
groups did not differ significantly. This is
unexpected and points to the risk of bias in
handling the treated patients during the pro-
tocol therapy. From a clinical point of view,
the different conditioning regimen used during
the first transplantation may have biased the
selection of patients with a good or a poor
response for later intense salvage and may
have influenced the improved overall survival.
Without doubt selection and enthusiasm for
salvage treatments represent an important
aspect of the global therapeutic strategy in
MM, particularly when relapse occurs after
autologous transplantation. 15 Such a bias
might explain the very late significant sur-
vival differences, first identified 4-5 years fol-
owing inclusion into the IFM study. 19

During the past decade, the advances in
treatment have been fast and furious. MM
patients have become a fertile testing ground
for a number of new antineoplastic agents.
Thalidomide, once infamous, has shown itself
as a potent immunomodulatory drug that
induces responses even in patients with ther-
apy-resistant disease. Thalidomide therapy is
a viable initial alternative to chemotherapy
when combined with dexamethasone. 16
Bortezomib, a proteasome inhibitor, has sub-
stantial single-agent activity against myelo-
ma. Studies are under way that incorporate
zreatment with these drugs early in the
course of the disease, as are trials with borte-
zomib in combination with other agents. 17
How such developments will alter therapy for
MM patients remains to be seen in the near
future. In this unclear situation we now see a
range of randomized trials evaluating new
drugs. In the near future, several ongoing
studies will clarify the role of these novel
agents, including thalidomide and its analogs,
bortezomib etc., in the context of autologous
stem cell transplantation. However, consoli-
dating trials such as that designed and ongo-
ing within NMSG, may be hampered by double
autologous transplantation strategies not yet
documented to have an effect on survival. In
conclusion, by combining our analysis and
recent results in the literature 16 it is likely that
double transplantation as initial strategy will
not improve the final outcome among most
patients with MM. Therefore, we recommend
consolidation trials studying the effect of new
drugs to be based on a single high-dose mel-
phalan 200 mg/m² conditioning supported by
autologous transplantation.

References
3. Barlogie B, Gahrton G. Bone marrow transplan-
tation in multiple myeloma. Bone Marrow
5. Child JA, Morgan GJ, Davies FE, et al. High-
6. Moreau P, Facon T, Attal M, et al. Comparison of 200 mg/m² melphalan and 8 Gy total body irradiation plus 140 mg/m² melphalan as con-
dose therapy and autologous peripheral stem cell transplantation in multiple myeloma: up-front or rescue treatment? Results of a mul-
10. Attal M, Harousseau J-L, Facon T, et al. Single vs double autologous stem-cell trans-
14. Lenhoff S, Martin Hjorth, Jan Westin, et al. Impact of age on survival after high-dose therapy with autologous stem cell support in newly diagnosed multiple myeloma: a popula-
tiple myeloma: clinical findings and method-
Hematology Reviews 2009; volume 1:e3

Tuberculosis associated thrombocytopenic purpura: effectiveness of antituberculous therapy

Raphael Borie,1,2 Claire Fieschi,2 Eric Oksenhendler,2 Lionel Galicier2
1Department of Pneumology A, Hospital Bichat, Assistance Publique, Hôpitaux de Paris, Paris, France
2Department of Clinical Immunology, Hospital St Louis, Assistance Publique, Hôpitaux de Paris, Paris, France

Abstract

Association of immune thrombocytopenic purpura and tuberculosis is a rare condition. In 5 patients presenting with this association, anti-tuberculous therapy was effective on both tuberculosis and thrombocytopenia suggesting a causal relationship between tuberculosis and immune thrombocytopenic purpura.

Introduction

Immune thrombocytopenic purpura (ITP) is characterized by a low platelet count associated with the presence of platelet autoantibodies. The diagnosis of ITP remains a diagnosis of exclusion, and a bone marrow examination should be performed in patients with atypical features. In 5-10% of cases, ITP is associated with chronic infection (HIV, HCV), systemic autoimmune disorders, lymphoproliferative disorders, and primary immunodeficiency. In adults, ITP is usually chronic (i.e. more than six months in duration). The response to treatment is defined as an increase in platelet count above 50×10⁹/L with at least a 2-fold increase in the initial value and remission is considered if platelet count reaches 150×10⁹/L.

We describe 5 cases of the association of tuberculosis and ITP and the effectiveness of anti-tuberculous therapy (ATT) on both tuberculosis and thrombocytopения (Figure 1).

Discussion

ITP may require treatment with oral prednisone or HD-IvIG. Response rates are almost 75%; however, only 20% of adult patients with ITP achieve persistent remission.1 In the present series, all patients had persistent or refractory ITP. No patient was cured at the time of tuberculosis diagnosis or within the month following the onset of ATT. However, within three months of ATT, ITP was in complete response in all patients. None required further ITP treatment or splenectomy.

Persistence of thrombocytopenia during the first month of ATT may be explained by the fact that Mycobacterium tuberculosis can remain alive in the first two months of anti-tuberculous therapy.2 These results suggest a direct relationship between tuberculosis and ITP.

ITP is a rare manifestation of tuberculosis. To our knowledge only 18 reports describing 27 cases of ITP associated with tuberculosis have been reported.4-8 All patients were treated with ATT in combination with corticosteroid, HD-IvIG or vincristine. In all cases platelet count recovered with ATT. The correction of thrombocytopenia in all patients within two months of ATT suggests a causal relationship between tuberculosis and thrombocytopenia, although the mechanism remains unknown.

Few causative conditions may bind thrombocytopenia to tuberculosis. The presence of an underlying disease causative of thrombocytopenia may favor tuberculosis. HIV infection is a well known cause of ITP and tuberculosis.1 Autoimmune disorders and lymphoproliferative diseases, that may cause ITP, are by themselves or when treated, immunosuppressive conditions.1 None of our patients had such an etiology.

Disseminated tuberculous infection may cause peripheric or central thrombocytopenia. Tuberculous splenic abcess is rare but a possible cause of hypersplenism and peripheric thrombocytopenia. None of our patients had splenomegaly. All patients had bone marrow aspiration to confirm peripheric thrombocytopenia, while bone marrow involvement of tuberculosis may provoke central thrombocytopenia. Hemophagocytic lymphohistiocytosis is an uncontrolled activation of T cells and macrophages, and an overproduction of inflammatory cytokines responsible for thrombocytopenia and anemia. It has been previously described secondary to tuberculosis.3 However, bone marrow aspirate excluded this diagnosis.

Appearance of thrombocytopenia after treatment of tuberculosis is classically induced by rifampicin and recovered after stopping rifampicin.10 On the contrary, development of tuberculosis after long-term steroid treatment is not uncommon while steroids are immunosuppressive. One of our patients (case 3) had received corticoid before the diagnosis of tuberculosis was made. At first she was asymptomatic and chest radiography was considered as normal. But four months later, she was coughing and dyspneic; chest radiography revealed abnormalities and Ziehl coloration of the sputum was positive for BAAR. The short

Patients were 3 men and 2 women (Table 1). Median age was 38 years. Major symptoms were pulmonary symptoms in 3 cases and bleeding in 2 cases. However, thrombocytopenia was symptomatic in 4 patients. In 4 of the 5 cases, clinical manifestations of tuberculosis and ITP were concomitant. In one case (patient 3), chest radiography abnormalities were detected four months after the diagnosis of thrombocytopenia. For each patient, ITP diagnosis was based on a platelet count below 30×10⁹/L and normal bone marrow examination. No patient had splenomegaly. Other secondary forms of ITP were excluded. All patients responded to high-dose intravenous immunoglobulin (HD-IvIG) as in most cases of ITP; however, none had complete and sustained response. In 3 patients, platelet counts remained below 30×10⁹/L despite corticosteroid and HD-IvIG. These patients had partial response to danazol or vincristine. Mycobacterium tuberculosis was identified in culture in 3 patients. Histological examination of an adenopathy confirmed the diagnosis in the 2 others. Each strain of mycobacterium tuberculosis was sensitive to conventional therapy. All patients completed treatment and were considered to be cured for tuberculosis. Within two months of anti-tuberculous therapy, all 5 patients were in ITP complete response and were off specific therapy for thrombocytopenia. Patient 2 required corticosteroid for three months for associated hemolytic anemia.

Case Reports

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course of steroids (less than a month) and the brief period between steroid treatment and the discovery of radiographic abnormalities suggest that active tuberculosis was already present when steroids were prescribed and that the treatment only favored progression of tuberculosis. In all other cases, active tuberculosis and thrombocytopenia were concomitant.

The pathophysiology of thrombopenia in tuberculosis remains unanswered. This is a rare condition, estimated to occur in less than 1% of cases of tuberculosis. *Mycobacterium tuberculosis* may share antigen with platelet leading to antiplatelet antibody formation. Specific HLA presentation of tuberculosis could also lead to antiplatelet immunity response in some patients.1 However, only one patient developed an auto-antibody. In this case, these were anti-hematia antibodies revealed with Coombs testing. None of our patients tested positive for antinuclear antibodies.

Tuberculosis is a rare but curative cause of thrombocytopenia. Thrombocytopenic patients, and particularly chronic and hard to treat ITP, should benefit from tuberculosis depistage. In cases of tuberculosis associated ITP, recurrence of thrombocytopenia is frequent in the first two months, and patients may benefit from close observation leading to a continuation of ITP specific therapy.

### Table 1. Characteristics of the 5 patients with immune thrombocytopenic purpura and tuberculosis.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>Delay between ITP and tuberculosis</th>
<th>Symptoms</th>
<th>CT scan</th>
<th>Diagnosis</th>
<th>Duration of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>56/M</td>
<td>None</td>
<td>Pulmonary</td>
<td>Tree in bud pattern bronchiolitis of the upper lobes</td>
<td>Sputum culture</td>
<td>6 months</td>
</tr>
<tr>
<td>2</td>
<td>16/F</td>
<td>None</td>
<td>Asthenia</td>
<td>Retroperitoneal adenopathy</td>
<td>Biopsy of the adenopathy</td>
<td>9 months</td>
</tr>
<tr>
<td>3</td>
<td>40/F</td>
<td>4 months</td>
<td>Pulmonary</td>
<td>Cavernous destruction of the left upper lung</td>
<td>Sputum culture</td>
<td>6 months</td>
</tr>
<tr>
<td>4</td>
<td>38/M</td>
<td>None</td>
<td>Fever</td>
<td>Pulmonary micronodules and mediastinal adenopathies</td>
<td>Biopsy of an adenopathy</td>
<td>6 months</td>
</tr>
<tr>
<td>5</td>
<td>26/M</td>
<td>None</td>
<td>Pulmonary</td>
<td>Cavernous destruction of both upper lobes</td>
<td>Sputum culture</td>
<td>6 months</td>
</tr>
</tbody>
</table>

F, female; M, male.

![Figure 1. Platelet counts in 5 patients presenting idiopathic thrombocytopenic purpura concomitant to tuberculosis. Thrombocytopenia resolved within three months of anti-tuberculous therapy.](image-url)
References

Cardiototoxicity of tyrosine kinase inhibitors in chronic myelogenous leukemia therapy

Zhenshu Xu,1 Shundong Cang,2 Ting Yang,3 Delong Liu1
1Division of Hematology and Oncology, New York Medical College, Valhalla, NY, USA
2Department of Hematology, Institute of Hematology, Union Hospital, Fujian Medical University, Fuzhou, China

Abstract

Emerging evidence suggests that the three tyrosine kinase inhibitors currently approved for the treatment of patients with chronic myelogenous leukemia (CML) – imatinib, dasatinib, and nilotinib – have potential cardiotoxic effects. The mechanisms behind these events, and the relations between them, are largely unclear. For example, relative to dasatinib and nilotinib, severe congestive heart failure and left ventricular dysfunction are rare but prominent with imatinib treatment, particularly in patients receiving higher doses (>600 mg/day). In comparison with imatinib, prolongation of the QT interval is relatively common in patients treated with either dasatinib or nilotinib. In contrast to nilotinib, pericardial effusions are observed with both imatinib and dasatinib. It is suggested that these data, an evaluation of cardiac status, use of concomitant medications, and potential risk factors should be considered in the management of CML.

Introduction

BCR-ABL tyrosine kinase is a key molecule responsible for the pathophysiology of chronic myelogenous leukemia (CML). Tyrosine kinase inhibitors (TKIs) directed against BCR-ABL are currently the cornerstone of treatment for patients with CML. Imatinib (Gleevec; Novartis Pharmaceuticals Corporation, New Jersey, USA) was the first TKI approved for CML. Imatinib has marked response and survival benefits over interferon-α plus low-dose cytarabine and is presently the only TKI licensed for first-line treatment.3,4 However, despite the remarkable success of imatinib, many patients discontinue treatment because of either resistance or intolerance to this drug.

In the pivotal phase III IRIS (International Randomized Study of Interferon and STI571) trial, primary resistance was observed in 24% of patients, and secondary resistance presented as relapse in 17% of patients and disease progression in 7% after 4.5 years.5 After six years of follow-up, 34% of patients had discontinued imatinib treatment, mostly (14%) because of an unsatisfactory therapeutic effect (defined as lack of efficacy/progression), but a number of patients (5%) also stopped receiving the drug as a result of adverse events (AEs) or abnormal laboratory values.6 Further treatment options therefore continue to be developed.

Second-line choices for treatment include increasing the dose of imatinib, or changing therapy to dasatinib [Sprycel®; Bristol-Myers Squibb Co. (BMS), New York, USA] or nilotinib (Tasigna®; Novartis Pharmaceuticals Corp., New Jersey, USA). Newer agents for imatinib failures are still under clinical development.7 Dasatinib potently inhibits BCR-ABL.8 Compared with imatinib, dasatinib has 325-fold greater activity against native BCR-ABL.9 Furthermore, dasatinib inhibits all imatinib-resistant mutations of this molecule (key mediators of imatinib resistance) except the T315I mutant, which is resistant to all currently available TKIs.10 It is, however, relatively insensitive to the F317L mutation.11 Dasatinib was originally approved at the dosage of 70 mg twice daily, following data from the START (the SRC/ABL Tyrosine Kinase Inhibition Activity: Research Trials of Dasatinib) program of clinical studies.10-12 In November 2007, the label for dasatinib was changed to include updated dosing information, safety information from more than 2,100 patients, and data from a randomized comparison with high-dose imatinib.13 The recommended starting dose for patients with chronic phase (CP) CML is now 100 mg once daily. The starting dose for advanced disease remains 70 mg twice daily.

Nilotinib (an analog of imatinib) is also active against all imatinib-resistant BCR-ABL mutations except T315I.14 However, nilotinib is relatively inactive against common mutations in the ATP-binding P-loop domain,1 and those at the F359 residue in the catalytic domain, of BCR-ABL.15 Patients harboring such mutations at baseline do not respond to nilotinib and progress quickly during treatment. Furthermore, patients who do not have these mutations at baseline may eventually develop them when resistance to imatinib occurs.16 Nilotinib has currently been approved by the FDA for treating imatinib-resistant and -intolerant patients with CP or accelerated phase CML, but not those suffering from blast crisis.

TKIs share a number of common adverse effects (AE), including neutropenia and thrombocytopenia. Cardiototoxicities of the TKIs have become a clinical concern. This review provides an overview of common AEs and cardiotoxicities associated with these TKIs.

Common adverse events associated with tyrosine kinase inhibitors

A number of AEs are associated with TKIs. The most common ones are hematologic. In the IRIS study, anemia, neutropenia, and thrombocytopenia (all grades) were each reported by 45-61% of patients receiving imatinib.1 Grade 3-4 anemia, neutropenia, and thrombocytopenia were observed in 3%, 14%, and 8% of patients, respectively. The updated dasatinib label includes data from the START-R trial, a comparison of dasatinib 70 mg twice daily with high-dose imatinib (800 mg/day) in patients with CP CML resistant to standard-dose imatinib.17 In this study, the incidences of grade 3-4 thrombocytopenia and neutropenia for high-dose imatinib were 14% and 39%, respectively. The START-R study also showed that dasatinib has significant response and progression-free survival benefits compared with high-dose imatinib, and that significantly more patients discontinue high-dose imatinib than dasatinib.18

The 100 mg once daily dose for dasatinib in patients with CP CML was approved as a result of data from a phase III dose modification study.19 Shah and colleagues compared dasatinib 100 mg once daily, 50 mg twice daily, 140 mg once daily, and 70 mg twice daily doses in CP CML. In this study, response rates were...
Cardiotoxicities observed in chronic myeloid leukemia

Current evidence suggests that TKIs have potential cardiotoxic effects. Cardiac AEs reported include palpitations, arrhythmia, QT prolongation, pericardial effusions, myocardial ischemia, myocardial infarction, and congestive heart failure (CHF). All of the clinically available BCR-ABL inhibitors report the potential for cardiotoxicity in their respective package inserts (Table 1).12,13,18

Imatinib

Although rare, severe CHF and left ventricular dysfunction have been reported during imatinib treatment, especially in patients with risk factors or comorbidities. In the IRIS trial, this was observed in 0.7% of patients.1,2,18 Cardiotoxicity associated with high-dose imatinib was not reported during the START-R study.11 In smaller studies of high-dose imatinib, existing CHF was exacerbated in one study (6% of patients), and was related to mortality in another (4% of patients).12,22

In a large review of patients enrolled in imatinib clinical studies (n=1,276), 1.8% of patients had symptoms suggestive of systolic heart failure. Most had risk factors for cardiac failure. In total, 0.6% of patients had cardiac events attributed to imatinib treatment. The authors concluded that CHF is rare during imatinib treatment.13 A similar incidence of CHF (1%) was reported in a nine-year retrospective review at a single institution.14 Reports of smaller studies also confirm that cardiac failure is a rare feature of imatinib therapy.21,22

The mechanism underlying imatinib-induced cardiac failure is currently unclear. In an in vitro study, physiological concentrations of imatinib significantly and adversely affected mitochondrial membrane potential, apoptosis, cell viability, and cellular ultrastructure.2,77 This cardiotoxic effect may be linked to inhibition of BCR-ABL. Imatinib was reported to cause stress-induced and dose-dependent mitochondrial changes in murine ventricular myocytes, which was reduced by re-engineering the imatinib molecule such that BCR-ABL inhibition was hampered.21-22 Nonetheless, the re-engineered molecule may have had altered activities besides reduced BCR-ABL inhibition.

A second cardiac AE associated with imatinib therapy is fluid retention manifesting as pericardial effusion. Grade 3-4 fluid retention reactions, which included pericardial effusions, were reported in 2% of patients in the IRIS study and in 6% of all other CML clinical studies.1,28 Frank pericarditis has been observed in <0.1% of patients receiving imatinib (all indications).2 Other cardiac AEs include tachycardia, hypertension, hypotension, flushing, and peripheral coldness, were each reported in 0.1-1.0% of patients.18

Precautions and general guidelines for dose adjustment for cardiac AEs associated with imatinib treatment are included in the prescribing information and have been summarized in Table 2. CML patients with existing cardiac disease or cardiac risk factors should be monitored and treated accordingly.21,22 Patients should also be weighed regularly and monitored for signs and symptoms of fluid retention. Unexpected weight gain should be investigated carefully, and treated appropriately.21-22 Significant fluid retention (local or general) can usually be managed by interrupting imatinib treatment and using diuretics or other supportive care.2 In severe cases of fluid retention, imatinib should be withheld until this is resolved.

Nilotinib

Nilotinib can cause QT prolongation and sudden death and carries a black box warning for these side effects.24 Nilotinib prolongs the QT interval in a concentration-dependent manner, and the common AEs occurred in 1-10% of all patients in clinical trials.1,24 In a study in healthy volunteers, nilotinib was associated with a maximum mean QT interval increase of 18 ms (adjusted for placebo).14 In imatinib-resistant patients with CML, nilotinib caused a maximum mean QT change of 10 ms from baseline; QT increase >60 ms and QT >500 ms associated with nilotinib were reported in 2.1% and <1% of patients, respectively. Sudden deaths were reported in 0.6% of patients in a clinical study, and at a similar frequency in an expanded access study. The early occurrences of some of these deaths relative to the start of nilotinib treatment suggest that ventricular repolarization may have contributed to their occurrence.19

Another common cardiac AE associated with nilotinib therapy is myocardial ischemia. In the pivotal phase II study, it was reported in 7% (21/321) of patients with CP CML receiving nilotinib as second-line treatment.31 Uncommon cardiac events (0.1-1.0% of all patients in clinical trials) include cardiac failure, angina pectoris, atrial fibrillation, pericardial effusion, coronary artery disease, cardiomegaly, cardiac murmurs, and bradycardia. Rare events (of uncertain frequency) include myocardial infarction, ventricular dysfunction, pericarditis, cardiac flutter, and extrasystoles.24

The mechanisms underlying these AEs are still unclear. In a manner similar to imatinib, nilotinib was found to decrease the cellular viability of rat cardiomyocytes cultured in vitro, although the integrity of the mitochondrial membrane potential was unaffected.27 However, nilotinib has also been found to inhibit human ether-a-go-go related gene (hERG) potassium currents with an IC50 of 0.66 μM. This concentration is approximately one-tenth the expected Cmax for this compound, well within therapeutic levels. This mechanism is likely to underlie nilotinib-induced QT prolongation. Inhibition of hERG channels is established as a cause of QT prolongation for a number of compounds, and is a significant barrier in the development of new drugs.32 Indeed, the phase II development of the aurora kinase inhibitor MK-0457 (VX-680) was recently suspended, pending a full analysis of all efficacy and safety data. The decision was based on preliminary safety data, in which QT prolongation was observed in one patient.33

The potential for QT prolongation and sudden death associated with nilotinib, although rare, necessitates vigilant monitoring. In particular, ECGs should be performed at baseline, seven days after initiation of treatment, periodically throughout therapy, and following dose adjustments. Electrolyte levels should be monitored periodically throughout therapy. Nilotinib is contraindicated for patients with hypokalemia, hypomagnesemia or long QT syndrome.24 The nilotinib prescribing information recommends dose adjustments for QT prolongation, presented in Table 2.

Dasatinib

The events of dasatinib-induced QT prolongation are rare although a warning for such a possible event is given. In single-arm studies of dasatinib, nine patients (1%) had QT prolongation reported as an AE.34 The mean QT interval increased by 3-6 ms (Fridericia's
method); this increase was not clinically relevant. In total, <1% of patients had a QT increase to >500 ms. In contrast with nilotinib, the IC50 for dasatinib for the inhibition of hERG currents (14.3 μM) is 100 times the expected Cmax for this drug. This may explain why QT prolongation is more clinically prominent for nilotinib than it is for dasatinib.

Common cardiac AEs (observed in 1 – <10% of all patients in clinical trials) include arrhythmia and palpitations. Severe pericardial effusions have been reported in 1% of all patients in all clinical studies, and the prescribing information for dasatinib includes a warning for this toxicity. Severe CHF has also been reported in 1% of all patients. In single-arm studies, CHF or ventricular dysfunction occurred in 4% (20/911) of patients. However, in the dose optimization study, dasatinib 100 mg once daily was not associated with any incidence of severe CHF or pericardial effusion in any patient; both AEs were reported in patients receiving the 70 mg twice daily dose (all grades, 4%; grade 3-4, 3%).

Both pericardial effusions and cardiac failure associated with dasatinib therapy may be caused by similar mechanisms to those associated with imatinib treatment, although, in contrast with imatinib, dasatinib has not been found to significantly affect mitochondrial membrane potential, apoptosis, cell viability, or cellular ultrastructure at physiological concentrations.

Uncommon cardiac AEs associated with dasatinib include angina pectoris, cardiomegaly, pericarditis, ventricular arrhythmia, and myocardial infarction (reported in Table 1. Reported incidence of cardiotoxicity during tyrosine kinase inhibitors treatment.

<table>
<thead>
<tr>
<th>Toxicity</th>
<th>n</th>
<th>Incidence (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Imatinib</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pericardial effusions</td>
<td>NR</td>
<td>6</td>
<td>Novartis</td>
</tr>
<tr>
<td>Systolic heart failure</td>
<td>1276</td>
<td>1.8</td>
<td>Atallah et al.</td>
</tr>
<tr>
<td>Congestive heart failure</td>
<td>553</td>
<td>0.7</td>
<td>Novartis</td>
</tr>
<tr>
<td>Left ventricular dysfunction</td>
<td>553</td>
<td>0.7</td>
<td>Novartis</td>
</tr>
<tr>
<td>Cardiac failure</td>
<td>NR</td>
<td>0.1-1.0</td>
<td>Novartis</td>
</tr>
<tr>
<td>Flushing</td>
<td>NR</td>
<td>0.1-1.0</td>
<td>Novartis</td>
</tr>
<tr>
<td>Hypertension</td>
<td>NR</td>
<td>0.1-1.0</td>
<td>Novartis</td>
</tr>
<tr>
<td>Hypotension</td>
<td>NR</td>
<td>0.1-1.0</td>
<td>Novartis</td>
</tr>
<tr>
<td>Peripheral coldness</td>
<td>NR</td>
<td>0.1-1.0</td>
<td>Novartis</td>
</tr>
<tr>
<td>Tachycardia</td>
<td>NR</td>
<td>0.1-1.0</td>
<td>Novartis</td>
</tr>
<tr>
<td>Pericarditis</td>
<td>NR</td>
<td>&lt;0.1</td>
<td>Novartis</td>
</tr>
<tr>
<td><strong>Dasatinib</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe pericardial effusions</td>
<td>NR</td>
<td>1</td>
<td>BMS</td>
</tr>
<tr>
<td>Congestive heart failure</td>
<td>911</td>
<td>4</td>
<td>Brave et al.</td>
</tr>
<tr>
<td>Arrhythmia</td>
<td>NR</td>
<td>1-&lt;10</td>
<td>BMS</td>
</tr>
<tr>
<td>Palpitations</td>
<td>NR</td>
<td>1-&lt;10</td>
<td>BMS</td>
</tr>
<tr>
<td>QT prolongation &gt; 500 ms</td>
<td>-300</td>
<td>&lt;1</td>
<td>BMS</td>
</tr>
<tr>
<td>Augia pectoris</td>
<td>NR</td>
<td>0.1-&lt;1</td>
<td>BMS</td>
</tr>
<tr>
<td>Cardiomegaly</td>
<td>NR</td>
<td>0.1-&lt;1</td>
<td>BMS</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>NR</td>
<td>0.1-&lt;1</td>
<td>BMS</td>
</tr>
<tr>
<td>Pericarditis</td>
<td>NR</td>
<td>0.1-&lt;1</td>
<td>BMS</td>
</tr>
<tr>
<td>Ventricular arrhythmia</td>
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<td>BMS</td>
</tr>
<tr>
<td>Acute coronary syndrome</td>
<td>NR</td>
<td>&lt;0.1</td>
<td>BMS</td>
</tr>
<tr>
<td>Myocarditis</td>
<td>NR</td>
<td>&lt;0.1</td>
<td>BMS</td>
</tr>
<tr>
<td><strong>Nilotinib</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myocardial ischemia</td>
<td>321</td>
<td>7</td>
<td>Kantarjian et al.</td>
</tr>
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<td>Increase in QTcF &gt;60 ms from BL</td>
<td>232</td>
<td>2.1</td>
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<tr>
<td>Palpitations</td>
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<td>Novartis</td>
</tr>
<tr>
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<td>NR</td>
<td>0.1-1</td>
<td>Novartis</td>
</tr>
<tr>
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<td>0.1-1</td>
<td>Novartis</td>
</tr>
<tr>
<td>Bradycardia</td>
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<td>0.1-1</td>
<td>Novartis</td>
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<tr>
<td>Cardiac murmur</td>
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<tr>
<td>Cardiomegaly</td>
<td>NR</td>
<td>0.1-1</td>
<td>Novartis</td>
</tr>
<tr>
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<td>0.1-1</td>
<td>Novartis</td>
</tr>
<tr>
<td>Pericardial effusion</td>
<td>NR</td>
<td>0.1-1</td>
<td>Novartis</td>
</tr>
<tr>
<td>Death</td>
<td>867</td>
<td>0.6</td>
<td>Novartis</td>
</tr>
</tbody>
</table>

BL, baseline; NR, not reported, representing all patients within each agent’s clinical trials. Possibly resulting from ventricular repolarization.
0.1 – <1% of patients). Rare events (reported in <0.1% of patients) include myocarditis and acute coronary syndrome.13

Dasatinib should be administered with caution to any patient at risk of cardiac problems (especially prolongation of the QT interval). Patients with pre-existing congenital long QT syndrome or those receiving anti-arrhythmic medicine should be monitored closely.13 Patients with hypokalemia or hypomagnesemia should have these conditions corrected prior to receiving dasatinib. As several anti-neoplastic agents, including the anthracyclines, are associated with cardiotoxicity, the treatment history of a patient should also be taken into consideration before commencing any therapy. Fluid retention events can typically be managed by supportive measures. Dose interruption is also indicated (Table 2).13

Conclusions

Although rare, severe CHF and left ventricular dysfunction have occurred with imatinib treatment, especially in patients receiving higher doses. Cardiac failure is also an uncommon feature of nilotinib and dasatinib therapy. However, CHF has not yet been reported in patients with CP CML receiving dasatinib with a starting dose of 100 mg once daily. Compared with imatinib, palpitations and prolongation of the QT interval are relatively common with both dasatinib and nilotinib. Nilotinib carries a mon feature of nilotinib and dasatinib therapy. Although rare, severe CHF and left ventricular dysfunction have occurred with imatinib treatment, especially in patients receiving higher doses. Cardiac failure is also an uncommon feature of nilotinib and dasatinib therapy. However, CHF has not yet been reported in patients with CP CML receiving dasatinib with a starting dose of 100 mg once daily. Compared with imatinib, palpitations and prolongation of the QT interval are relatively common with both dasatinib and nilotinib. Nilotinib carries a mon feature of nilotinib and dasatinib therapy.

References


Table 2. Precautions and dose modifications on the emergence of cardiac events during tyrosine kinase inhibitors treatment.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose modification</th>
<th>Precautions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imatinib</td>
<td>On the emergence of a severe event, withhold until the event has resolved. Resume at an appropriate dose, depending on initial severity of event.</td>
<td>Carefully monitor any patient with or at risk of cardiac failure. All patients with signs or symptoms of cardiac failure should be evaluated and treated.</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>On the emergence of a severe event, withhold until the event has resolved or improved. Resume at an appropriate dose, depending on initial severity of event.</td>
<td>Administer with caution to patients with or at risk of QTc prolongation: those with hypokalemia, hypomagnesemia, or congenital long QT syndrome; or taking medicines known to prolong QT, including anti-arrhythmic drugs, and cumulative high-dose anthracycline therapy. Correct hypokalemia or hypomagnesemia prior to administration.</td>
</tr>
<tr>
<td>Nilotinib</td>
<td>QTc &gt;480 ms: 1) withhold therapy, correct serum potassium and magnesium levels if below normal, and review concomitant medication; 2) resume at prior dose in &lt;2 weeks if QTc returns to &lt;450 ms and &lt;20 ms of baseline; 3) reduce dose to 400 mg/day if QTc 450-480 ms after 2 weeks; 4) discontinue if QTc returns to &gt;480 ms after dose reduction; 5) repeat ECG assessment approx. 7 days after any dose adjustment.</td>
<td>Do not administer to patients with long QT syndrome. Do not administer drugs known to prolong QT, and strong CYP3A4 inhibitors. Correct hypokalemia or hypomagnesemia prior to administration, and periodically monitor serum electrolyte levels during therapy. Perform ECGs at baseline, 7 days after treatment starts, periodically as indicated clinically, and after any dose adjustment.</td>
</tr>
</tbody>
</table>
than T3151 during nilotinib therapy after imatinib failure and are associated with progression in patients with Ph-positive leukemia. Haematologica 2007;92(S1): 337.


Bacterial contamination of platelet concentrates: pathogen detection and inactivation methods

Dana Védy, Daniel Robert, Danielle Gasparini, Giorgia Canellini, Sophie Waldvogel, Jean-Daniel Tissot
Service Régional Vaudois de Transfusion Sanguine, Lausanne, Switzerland

Abstract

Whereas the reduction of transfusion related viral transmission has been a priority during the last decade, bacterial infection transmitted by transfusion still remains associated to a high morbidity and mortality, and constitutes the most frequent infectious risk of transfusion. This problem especially concerns platelet concentrates because of their favorable bacterial growth conditions. This review gives an overview of platelet transfusion-related bacterial contamination as well as on the different strategies to reduce this problem by using either bacterial detection or inactivation methods.

Introduction

Reduction of transfusion related viral transmission such as HIV, HBV or HCV has been a priority for blood transfusion services. Implementation of antibody screening followed by nucleic acid amplification techniques (NAT) introduced in blood donor screening during the last decade, have largely contributed to this reduction.1,5 Nevertheless, all blood products remain under the threat of emerging blood-transmitted infections (virus, protozoans, helminths, bacteria, prions)1 and bacterial transfusion related transmission is still associated to a high morbidity and mortality.1,3,15-18 Platelets are especially affected by this risk, and septic infections are most commonly associated with platelet transfusion because of the favorable bacterial growth conditions that are: i) room temperature storage allowing the growth of even small bacterial inoculums and, ii) the biological composition of platelet concentrates.5,10,11

The estimated rate of bacterial platelet contamination is about 1/2,000-3,000 units (whole-blood and apheresis-derived platelets)5,11 and a severe sepsis may be associated with one out of 6 of contaminated platelet units transfused.3 The risk of bacterial contamination of platelet concentrates has been estimated to be 50-250 times higher than the combined risk of HIV, HBV, HCV and HTLV-1/2.7 Transfusion related sepsis is often not recognized, and the real clinical and fatal prevalence are probably underestimated.19

Platelet transfusion-related bacterial contamination: some data

In the United States, bacterial contamination is considered, after transfusion errors, as the second most common cause of death related to transfusion.1 As mentioned by Holme et al., the estimated number of patients who received bacterial contaminated platelet concentrates per year ranges from 2,000-4,000, resulting in 200-600 cases of clinical sepsis, and an estimated 40-533 fatalities.2 The death risk in the USA has been estimated at 1/500,000 platelet concentrates. Between 1995 and 2001, the English hemovigilance system reported 21 transfusion-related bacterial contamination, leading to 6 deaths, in which 5 were attributed to platelet contamination.11 In France, Fournier-Wirth et al. reported that the incidence of transfusion related reactions due to bacterial contaminated platelets was about 1/25,000 units that was associated with severe morbidity or even mortality in about 3 cases each year in the period 1999-2003.15 The death rate was estimated to be one death per 200,000 distributed platelet concentrates.31

Platelet contamination: source and species of bacteria

Several mechanisms may lead to platelet contamination, the major being contamination by the skin flora at the site of puncture.3,7 Pathogens are essentially gram positive bacteria like Staphylococcus aureus, coagulase negative Staphylococci, viridans group Streptococci, Bacillus spp., Corynebacteria as well as anaerobic diphteroid gram positive bacilli such as Propionibacterium acnes.3,8,11 In 2004, in a review dealing with transfusion-transmitted bacterial infections, Wagner reported that about 56% of bacteria detected in platelet units implicated in clinical situations of transfusion associated sepsis were aerobic gram-positive bacteria.18 However, in the presence of gram negative organisms, the outcome was more frequently fatal (60%) when compared with gram positive ones (40%). The distribution of bacteria found in cases of transfusion related sepsis showed Staphylococcus spp. in 42% of cases, Escherichia coli in 9%, Bacillus spp. in 9%, Salmonella spp. in 9%, Streptococcus spp. in 12%, Serratia spp. in 8%, Enterobacter spp. in 7%, and other organisms in 4%. The clinician should be aware that uncommon pathogens might be encountered in platelet preparations.41,43

Infrequently, donor bacteremia may be present during blood collection, also leading to platelet contamination. In a large study evaluating the risk of septic platelet transfusion reactions, 23 septic transfusion reactions were observed; 15 out of 23 reactions (62.5%) were attributed to skin flora contamination, and 8 out of 23 (34.8%) resulted from bacteria, more likely associated to transient donor bacteremia.19 Furthermore, the authors also showed that pooled platelet concentrates from buffy coats have a higher bacterial contamination rate compared to apheresis platelets. A total of 32,333 pooled-platelet concentrates and of 134,159 single-donor platelet concentrates were transfused with a rate of septic transfusion reaction of 13/32,333 and 10/134,159, respectively. Thus, they observed a 5.4 fold increased rate with pooled-donor platelets.29 In an AABB Educational Session in Transfusion Medicine, Hillyer et al. reported that the prevalence of the bacterial contamination of whole blood derived platelets was 33.9 per 100,000 units compared to apheresis platelets whose prevalence was 51.0 per 100,000 units.3,13,17

The effect of bacterial screening of apheresis platelets has been evaluated. Eder et al. pointed out that while septic reactions associated with platelet transfusion were estimated to occur in about 1,250,000 transfusions, the rate of contaminated platelets determined after implementation of detection techniques was about 1,200 to 13,000. The authors hypothesized that low-level contamination was not necessarily associated with a clear clinical response, and that more particularly, neutropenic and febrile patients were often under antibiotic therapy that may change the clinical picture.40
Platelet transfusion-related bacterial infection

The occurrence of a severe bacterial infection related to platelet transfusion has been correlated to bacterial proliferation in platelet concentrates, and a bacterial contamination $>10^4$ CFU/mL has been considered as a serious infectious risk. In 2006, Yomtovian et al. reported data from a surveillance program for detection of bacterial contamination of platelets in a university hospital from 1991 to 2004. These data concern passive surveillance (transfusion reaction-triggered) and active surveillance (prospective methods). During the surveillance period, 216,283 platelet units were transfused. Only one type of bacterial contamination was detected by the passive surveillance method (Pseudomonas aeruginosa, 3-day old random-donor platelets). Thirty-eight contaminated platelet units or pools were detected by active surveillance. Six of them were not transfused. When transfused, the same contaminant was isolated from blood cultures obtained from the recipient in 7 cases. Coagulase-negative staphylococci was the most frequently isolated bacteria species followed by Staphylococcus aureus. Thirteen of the patients who received the 32 contaminated platelets (31 detected by active surveillance and one detected by passive surveillance) developed transfusion reactions. Transfusion reaction rate was significantly higher for pooled random-donor platelets. Severe transfusion reactions occurred in 9 cases, and 3 patients died of complications, likely to be associated with the transfusion of contaminated platelet units (Pseudomonas aeruginosa, Staphylococcus aureus and Serratia marcescens). Nine of the 13 transfusion-reactions were associated with bacterial counts $>10^4$ CFU/mL. The virulence of the bacteria appeared to be more important then the bacterial load. However, transfusion reactions with fever, rigors and hypotension were observed with coagulase-negative staphylococci at bacterial counts as low as $10^2$ CFU/mL. As bacterial growth increased over time, platelet units, were older, the risk of high bacterial amounts was higher, and consequently there was a higher risk of sepsis. In 1984, the FDA allowed the extension of platelet storage from five to seven days. This decision led to an increased rate of platelet transfusion-related infections, directly associated with the oldest transfused units. Therefore, in 1986, the storage was reduced from seven to five days.

As mentioned above, severity of the clinical manifestations related to contaminated platelet units are determined by quantitative/ qualitative parameters (number of CFU infused, type of bacteria, rate of proliferation, latency phase). Whereas in some cases fever and chills are present during the transfusion, in many cases the patient remains asymptomatic. In the symptomatic recipient, fever and chills may be observed within two hours after the start of transfusions. Hypotension, nausea, vomiting, diarrhea, oliguria, respiratory symptoms and shock may be observed. The severity of a septic transfusion reaction also depends on the patient’s characteristics and may be more severe in immunocompromised patients.

In order to reduce the risk of post-transfusion sepsis from bacterial contaminated platelets, various procedures have been implemented: improved donor selection, single-donor apheresis platelets, reduction of the contamination risk of the phlebotomy process by optimal skin disinfection procedures and the removal of the first 10-30 mL of the initial collected blood, storage time limitation, bacterial detection and inactivation methods. Indeed, as the major cause of platelet contamination is the normal skin flora at the site of puncture, optimal skin disinfection may significantly reduce contamination. However, when the collection needle enters the skin, bacteria may be introduced into blood. Different studies have suggested that the removal of the first aliquot of the initial collected blood may reduce the risk of bacterial contamination in blood donation.

Methods for detection of bacterial contamination of platelets: culture methods

When compared with bacterial detection methods, surrogate tests such as pH measurement, glucose level determination or Gram’s stain coloration have been shown to have low sensitivity and thus, can not be recommended. Two methods have been approved by the FDA for the detection of bacterial contamination in platelets. The first one is BacT/ALERT® (BioMérieux Inc.), an automated colorimetric blood culture method, based on the detection of carbon dioxide produced by proliferating microorganisms, which allows the detection of both aerobic and anaerobic bacteria as well as yeasts and fungus. The second one is eBDS® (Pall Corporation), an enhanced bacterial detection method based on the measurements of oxygen consumption by bacteria in the milieu. A comparison between the advantages and disadvantages of both methods is presented in Table 1 [adapted from (4)]. The Verax Biomedical Platelet PGD® Test for Bacterial detection (Verax Biomedical, Inc.) is a new rapid and qualitative immunoassay test for the detection of aerobic and anaerobic bacteria in leukocyte reduced apheresis platelets. However, in the absence of sufficient data, a comparison between this method and both BacT/ALERT® and Pall eBDS® is not possible.

BacT/ALERT® is an automated colorimetric blood culture method allowing the detection of both aerobic and anaerobic bacteria as well as yeasts and fungal microorganisms, in two culture bottles, one for aerobic and one for anaerobic pathogens. The bottom of the bottles contains a pH sensitive liquid sensor which changes its color according to the amounts of CO$_2$ released. Whereas bacteria produce CO$_2$, yeasts and fungi are high acid-producing organisms. Inoculation of approximately 7.5 mL of the platelet sample is necessary for each bottle, and is performed 24 hours after collection. The bottles are then incubated at 37°C. The CO$_2$ production is correlated with the alteration of the reflection of the light on the sensor. When the color changes, an alarm is set off. The bottles are continuously analyzed and tested for up to 5-7 days. This system has been used for routine detection of platelet bacterial contamination in the Netherlands, Belgium and Wales for several years.

In 2002, McDonald et al. reported data resulting from the evaluation of the BacT/ALERT® system after inoculation, in 2-day old apheresis platelets, of different bacterial species (9 gram positive, 5 gram negative) and one fungal microorganism at a concentration of 10-100 CFU/mL. The results were compared to thiglycollate broth cultures. The mean time for the bacterial detection ranged between 9.1-48.1 hours, with the exception of Propionibacterium acnes (89-177.6 hours). Analogous results were obtained by Brecher et al., who also confirmed the large delay required for detection of Propionibacterium acnes. However, it is important to note that anaerobic Propionibacterium acnes has a poor growth in the aerobic platelet conditions, and that its clinical relevance is all but clear. As bacterial load may be very low in freshly collected platelets (<1 CFU/mL) a storage period ≥24 hours is required before inoculation, in order to allow bacterial growth and to avoid false negative results.

To better assess the prevalence and nature of bacterial contamination of pooled and apheresis platelets, the GERMS (German Evaluation of Regular Monitoring Study) Group of the Red Cross Transfusion Services initiated a prospective multimember study including 9 different centers. The results of this study have been reported by Schrezenmeier et al. in 2007. The BacT/ALERT® method was used to analyze 52,243 platelet concentrates (15,198 collected by apheresis and 37,045 pooled platelets). Of the 282 platelet concentrates with a first positive culture, a
Two cases of life-threatening sepsis due to Serratia marcescens were reported in 43% of them, with bacteria isolated were skin flora bacteria. Propionibacterium acnes was found in 54% of them, and staphylococci species in 43% of them, with Staphylococcus epidermidis in most of the cases. Serratia marcescens and Staphylococcus aureus, which are potentially pathogenic bacteria, were isolated respectively in one plasma pooled platelet concentrate and in one apheresis platelet concentrate. This study demonstrated that a large scale routine screening of platelets prepared by different blood centers was feasible. However, the authors observed that such a screening did not allow the prevention of the transfusion of contaminated units because of the interval of time needed after inoculation and the detection of the contaminant. In addition, the risk of false negative results was not eliminated by this approach.3

Two cases of life-threatening sepsis due to Bacillus cereus contamination despite the BacT/ALERT® detection method were described by te Boekhorst et al. in the Netherlands.25

In another important multi-center German study, comparing three bacterial detection methods under routine conditions, Schmidt et al. reported 2 severe transfusion reactions after transfusion of 2 split apheresis platelet concentrates contaminated with Klebsiella pneumoniae, despite a negative screening with both BacT/ALERT® and Pall eBDS® detection methods.4

In the Netherlands, te Boekhorst et al. reported their experience with the BacT/ALERT® system implemented in their center for routine bacterial screening of platelets in October 2001. Over a period of two years, 28,104 pooled platelets were screened: 203 of them were detected as being contaminated and 125 out of 203 had already been released at the time of the detection. Ninety percent of them were already transfused. No adverse reactions such as fever, hypotension, or other unexplained clinical deterioration, were observed after a contaminated transfusion. Early detection (<48 hours) of bacterial contamination with microbiological confirmation was possible in 59 concentrates, and 48 of them could be recalled. For late detection (>48 hours), only 33 of 125 contaminated concentrates could be recalled. The authors also observed that 68% of the BacT/ALERT® screening cultures became positive after an incubation of 48 hours.7 They noted that this low rate was in contrast with several studies that showed higher rates of bacterial detection after an incubation of 48 hours. For instance, Wagner and Robinette showed that deliberate bacterial inoculation in platelet concentrates could be detected after 48 hours in all of the cases.12,13 Brecher et al. reported detection of inoculated microorganisms in apheresis platelet units (with the exception of Propionibacterium acnes) in a median time of 9.3-18.9 hours (10 CFU/mL) or 8.7-18.2 hours (100 CFU/mL).21,22 Te Boekhorst et al. pointed out the fact that this difference could be explained by a presumably very low bacterial load in their platelet concentrates compared to the experimental inoculation load.10

As previously mentioned, Pall eBDS® is a culture method system based on the bacterial consumption of oxygen and only allows detection of aerobic and facultative anaerobic bacteria. Twenty-four hours after collection, 3 mL of the platelet concentrate are filtered in order to remove platelets and leukocytes, and then are inoculated into a sampling bag. The pouch contains two tablets: i) a sodium polysulphonate sulphonate dissolvable tablet that reduces the natural inhibitors of bacterial growth and also acts as a platelet aggregating agent in order to lower platelet competitive consumption of O2; and ii) a trypticase soy medium-containing tablet that enhances the sensitivity by providing nutrients for bacterial growth. The sample is incubated for 24 hours at 35°C, and the level of O2 is measured.24,25,26

Like BacT/ALERT®, the Pall eBDS® in vitro sensitivity was determined to be between 1-10 CFU/mL. Moreover, in order to reduce the risk of false negative results, a period of storage ≥24 hours has been advocated.25 The performance of the Pall eBDS® was evaluated in four test sites after inoculation 1-15 CFU/mL of 10 different bacterial species known to be associated with fatal platelet transfusion related outcome. The inoculated samples were transferred in Pall eBDS® bags immediately after inoculation or after a 24 hour storage at 22°C. The results were reported by Holme et al. who showed that all samples incubated 24 hours after inoculation were detected as being contaminated (100% sensitivity) and no false positives were obtained with 713 uninoculated platelets.2

Fournier-Wirth et al. reported the results from a study performed in 3 transfusion centers in France on pooled and apheresis platelets. The aim of this study was to evaluate the ability of the Pall eBDS® system to detect bacterial contamination after a reduction of the incubation time. The results were compared with the BacT/ALERT® system, considered in this study as a reference. Low levels (5-50 CFU/mL) of 5 different strains of bacteria were inoculated in the platelet units. The platelets were stored at 22°C, 24 hours before

| Table 1. A comparison of the advantages and disadvantages of BacT/ALERT® and Pall eBDS®, according to the data reported by Schmidt et al.* The sensitivity, efficiency and manageability of BacT/ALERT® and Pall eBDS® were compared in a multicenter-study carried out in 4 German centers. It concerned 6,307 pooled platelets and 4,730 apheresis. |
|---------------------------------|---------------------------------|
| **Advantages**                  | **Disadvantages**               |
| Sensitivity*                    | Detection of aerobic and only facultative anaerobic bacteria |
| Good specificity**              | A punctual measure in time whereas platelets may be transfused until 5 days after collection |
| Closed system                   | Platelets may be released before the time of detection |
| Easy and quick handling         | **BacT/ALERT®**                 |
|                                 | Detection of aerobic and anaerobic bacteria, yeasts, and fungal microorganisms |
|                                 | The bottles are continuously analyzed and tested, for up to 5-7 days |
|                                 | Easy and quick handling         |

*This evaluation revealed a better sensitivity for the BacT/ALERT® system, identifying 4 positive samples that were missed with the Pall eBDS®. **The specificity of BacT/ALERT® (0.027%, 28 out of 11,037 tested samples) was significantly lower compared to Pall eBDS® (0.003%, 3 out of 11,037 tested samples).
Pathogen inactivation

Pathogen reduction technologies allow inactivation of viruses and bacteria in contaminated platelet concentrates. Two main different approaches have been described.

Psoralen based method

The INTERCEPT blood system® (Cerus Corporation) uses amotosalen which is a synthetic psoralen, an organic compound found in fruit and vegetables like limes and celery. Amotosalen compound has the potential to penetrate into cells, to cross the nuclear membrane, and to reversibly intercalate into helical regions of nucleic acid. Exposure to a long-wave-length ultraviolet light (UVA, 320-400 nm) leads to covalent crosslinks between amotosalen molecules and pyrimidine bases, blocking DNA and RNA replication (cells and pathogens with nucleic acid genomes). After light treatment, the residual amotosalen as well as its metabolites are removed by a compound adsorbing device during a prolonged incubation. Platelets do not have nuclei and are not affected by psoralens. In 2005, Lin et al. reported results that advocate the efficiency of such an approach for virus inactivation. The authors studied 10 different families of viruses including: i) the most relevant for blood transfusion like HIV-1, HIV-2, HBV, HTLV-1, HTLV-2 or CMV, ii) viruses of emerging interest like parvovirus B19, West Nile virus, severe acute respiratory syndrome-coronavirus, and vaccinia virus, and, iii) model viruses like duck hepatitis virus (an HBV model), bovine viral diarrhea virus (an HCV model), bluetongue virus, feline conjunctivitis virus, simian adenovirus 15 and porcine parvovirus. According to the FDA, the process was defined as being effective if the pathogen load was reduced by 6-10 logs. The results of this study showed a significant log reduction of enveloped viruses that were uniformly sensitive to inactivation. The non-enveloped viruses showed a variable sensitivity to inactivation. Parvovirus B19 and human adenovirus 5 were inactivated to the limit of detection, whereas the other non-enveloped viruses were resistant to inactivation. In 2006, Singh et al. reported that the photochemical treatment of plasma with amotosalen inactivates high levels of gram positive (Streptococcus epidermidis) and gram negative (Klebsiella pneumoniae, Yersinia enterocolitica) bacteria. They determined that the mean log reductions achieved were >7.3 for Streptococcus epidermidis and Yersinia enterocolitica and >7.4 for Klebsiella pneumoniae. Photochemical treatment was also efficient at high initial titers on spirochetes with mean-reductions >5.9 for Treponema pallidum and >10.6 for Borrelia burgdorferi and on protozoa with mean-reduc-
comparable, the transfusion of treated platelets was associated with a lower platelet where the hemostatic effect of both photo-platelets and 26 of them did not require photochemical treated platelets, 32 control included 671 thrombocytopenic patients needed a greater proportion of treated platelet that con-

seem to be conserved.32,38-40 treated platelet concentrates and their viability has the potential to reduce the incidence of leukocyte mediated adverse immune reactions associated with platelet transfusion like trans-

fusion associated graft-versus-host disease and platelet related febrile non-hemolytic transfusion reaction.12,33,37Platelet concentrates and platelet related febrile non-hemolytic associated with platelet transfusion like trans-

fusions as secondary efficacy ber of platelet transfusion incidence of platelet number of platelet transfusion reactions, number of days to next platelet transfusion, num-

ber of platelet transfusion reactions, number of days bleeding, number of days of WHO grade 2 bleeding, number one hour and 24 hour platelet count increments, corrected count increments, num-

ber of days to next platelet transfusion, number of platelet transfusion incidence of platelet refractoriness and number of erythrocyte concen-

trate transfusions as secondary efficacy end points. Safety end points were defined by the number of platelet transfusion reactions, development of antibody to potential amotosalen neoantigens and overall safety. The study included 671 thrombocytopenic patients needing platelet transfusion support: 318 received photochemical treated platelets, 327 control platelets and 26 of them did not require platelet transfusion. This trial showed that whereas the hemostatic effect of both photo-

chemical treated and control platelets were comparable, the transfusion of treated platelets was associated with a lower platelet count increment after transfusion. This lower platelet count increment could partly be explained by the lower mean platelet dose in the photochemical treated group (3.7×10^11 vs. 4×10^11) in the control group; p<0.01 and by a greater proportion of treated platelet that con-

tained <3×10^11 platelets. Thus, patients who received photochemical treated platelets received more platelet transfusions and had a shorter interval between transfusion than patients who received conventional platelets.41

Studies performed in animals and humans reported no evidence for a toxicity of psoralen treatment.29,31-34 Webert et al. related acute toxic-

ity with amotosalen after UVA activation in rats with a lethal threshold dose of 150 mg/kg. In dogs, central nervous system alterations were observed after a threshold dose of 30 mg/kg. These doses were respectively 150,000, and 30,000 fold higher than the dose used for pathogen inactivation. Reproductive toxicity, determined by histological rather than functional evaluation, was observed at a threshold of 0.35 mg/kg, which was 350 times higher than the dose used for pathogen inactivation. Moreover, all substances used for pathogen inactivation are water soluble and rapidly excreted avoiding the bioaccumulation of trace amounts in treated blood products.22 In addition, transfusion of platelets or plasma treated with this method did not appear to induce adverse immunological response when evaluated by searching the presence of neoantigens.20,21

In 2005, Lin et al. evaluated the potential of photochemical treatment with amotosalen to create neoantigens. They quantified the amounts of residual amotosalen and photo-

products in photochemical treated platelets and plasma. Patients’ serum samples from 7 Phase III clinical trials including 523 patients who received more than 8,000 units of treated platelets or plasma, were assayed by enzyme linked immunosorbent assay (ELISA) for anti-

bodies to amotosalen neoantigens. The results indicated that no neoantigens were detected by ELISA after photochemical treatment with amotosalen.29

Riboflavin based method

The Mirasol® PRT (Navigant Biotechnologies Inc.) system is similar to the psoralen-based method, but uses a different photo-sen-
sitizer, riboflavin (vitamin B2) instead of psoro-

alen. Riboflavin is a natural component found in food (milk, beer, eggs, yeasts, leafy vegeta-
bles), and is classified as a “Generally-

Regarded-As-Safe” compound by the FDA.24,44,45 Riboflavin interacts with nucleic acids after exposure to UV light (280-360 nm) and causes irreversible damage to DNA/RNA (direct electrone transfer, production of singlet oxygen and production of hydrogen peroxide leading to the formation of hydroxyl radicals). A compound adsorbing device removal process for residual riboflavin metabolites may not be neces-
sary.44 Perez-Pujol et al. have studied the impact of this method on the functional and biochemical characteristics of platelet concen-

trates. They observed, with flow cytometry studies, the same changes in treated and non-
treated platelets, without modification of FvW, fibrinogen and FVa levels after five days of storage. They also showed that treated platelets have adhesive and cohesive functions similar to non-treated platelets.46 Ruane et al. have shown that the Mirasol® PRT system is able to inactivate viruses and bacteria in platelet concentrates. They observed significant log reductions for cell-associated and cell-

free HIV, West Nile virus, porcine parvovirus, and for gram positive and gram negative bacte-

ria such as Staphylococcus epidermidis or Escherichia coli. The authors noted that the platelet pH and the lactate production rate were, as suggested by the literature data, predictive of recoveries in 50.8-59.8%. Therefore, they used platelet pH and lactate production rate as platelet quality indicators and concluded that platelet cell quality was maintained after treatment and during storage.44 Goodrich et al. reviewed Mirasol® PRT performances and confirmed that this system was able to inacti-

vate viruses and various bacterias such as Staphylococcus epidermidis, Staphylococcus aureus, Escherichia coli, Pseudomonas aerugi-

nosa, Bacillus cereus, Serratia marcescens. These data suggested a significant reduction of the risk of platelet related bacteria transmis-

sion.24,44,45 Kumar et al. showed an increased genomic DNA degradation in leukocytes and bacteria after riboflavin and UV light treat-

ment, also suggesting that Mirasol® PRT tech-

nology may be an alternative to gamma irradiation to prevent transfusion associated graft-

versus-host disease.46

In December 2007, Klein et al. reported in a consensus conference about pathogen inactiva-
tion (Toronto, March 2007) that pathogen inac-
tivation methods should be implemented as soon as feasible and safe methods to inactivate a broad spectrum of infectious agents are available.47 The authors established a list of existing criteria and procedures that should be changed in case of implementation of pathogen inactiva-
tion such as the suppression of screening tests for: i) Treponema pallidum; ii) agents of low infectious titer and destroyed early by pathogen inactivation like West Nile virus, which is actu-

ally systematically tested in the US and Canada; iii) agents that are sensitive to pathogen inactiva-
tion for which redundant safety measures are taken (CMV, HTLV, HbsAg and those for which the methods of detection available nowadays lack specificity and sensitivity like those used to detect bacteria). Moreover, gamma irradiation of blood components performed to eliminate the risk of transfusion associated graft-versus-host disease could be eliminated. The authors con-

clude that an agent that is known to be ade-

quately inactivated by these technologies should not require screening tests unless of an unusually high infectious titer.42

[page 26] [Hematology Reviews 2009; 1:e5]
Towards a change of paradigm: inactivation versus detection

The ability of pathogen reduction technologies to inactivate a broad spectrum of organisms (virus, fungus, bacteria, parasites) is one of the most convenient answers to face the rapidly evolving epidemiological environment as well as the continuous appearance of new pathogens. Multiple different factors effectively contribute to the occurrence of emerging or re-emerging infectious agents: migration, travel, conflicts, climatic changes, demography, and numerous less trivial factors like for instance pet trade through e-business.\textsuperscript{3,4,6,7} The occurrence of pathogens with a strong epidemic potential, and/or with high prevalence, as well as the diversity of existing pathogens that are not systematically detected using standard screening approaches, strongly argue for the introduction of inactivation procedures rather than continuously introducing new biological tests, each being characterized by its own sensitivity and specificity.

Conclusions

The bacterial transmission still constitutes a significant problem in transfusion medicine. Because of their favorable bacterial growth conditions (storage at room temperature, biological composition) platelets are of special concern. As shown in this review, data available suggest that bacterial detection with the technology available nowadays may present a false sense of security. To our knowledge, there is no recently approved “revolutionary” bacterial detection technology that can dramatically and definitively make platelet transfusion safe. By contrast, pathogen inactivation has demonstrated its efficiency. However, long-term studies are still needed to demonstrate the safety of this approach. Newer pathogen inactivation technologies are currently under development, like CryoFacet red blood cell and platelet technology using counterflow elutriation to remove infectious agents in plasma, followed by ozonation and treatment with germicidal UV before releasing.\textsuperscript{22} Other approaches such as “glyco-engineering” the platelets in order to allow their cold storage and rapid bacterial detection (at the moment of release) are under development.\textsuperscript{23,24}

In summary, this review describes recent technologies that may be used to make platelet transfusion safe, with a particular emphasis on the prevention of bacterial contamination. Based on the literature review, and more particularly for people who are not directly involved in the development and/or implementation of these particular technologies (like the authors of this review), the choice between all these different approaches is very difficult.

References

Human heart-type fatty acid-binding protein as an early diagnostic marker of doxorubicin cardiac toxicity

Ashraf H. ElGhandour,1 Manal El Sorady,1 Sahar Azab,2 Mohammed EI Rahman1
1Hematology Unit, 2Cardiology Department, Faculty of Medicine, Alexandria University
1Military Medical Academy, Alexandria, Alexandria, Egypt

Abstract

Progressive cardiotoxicity following treatment with doxorubicin-based chemotherapy in patients with non-Hodgkin’s lymphoma (NHL) may lead to late onset cardiomyopathy. So, early prediction of toxicity can lead to prevention of heart failure in these patients. The aim of this work was to investigate the role of H-FABP as an early diagnostic marker of anthracycline-induced cardiac toxicity together with brain natriuretic peptide (BNP) as an indication of ventricular dysfunction in such patients. Our study was conducted on 40 NHL patients who received 6 cycles of a doxorubicin containing chemotherapy protocol (CHOP), not exceeding the total allowed dose of doxorubicin (500 mg/m²). Ten healthy controls were included in our study. Human heart-type fatty acid-binding protein (H-FABP) was assessed 24 hours after the first cycle of CHOP. Plasma levels of BNP were estimated both before starting the first cycle of chemotherapy were excluded.

Patients’ age ranged from 14 to 56 years with a mean of 36.2±10.02 years. Twenty-two of them were males, while 18 were females. Ten age and sex-matched healthy controls with no history of cardiac problems were included in our study. None of our patients had symptoms or signs of cardiac disease at presentation, nor had they undergone previous cardiac surgery. Patients with severe hepatic or renal disease or other medical conditions in which combination chemotherapy may be contraindicated were excluded from our study. Also, patients with early stage disease (stage I and non-bulky stage II) who are candidates for involved-field irradiation in addition to chemotherapy were excluded.

At presentation, full history and clinical examination, complete blood picture, bone marrow examination and lymph node biopsy were carried out in all patients. Routine laboratory investigations, ECG and echocardiography were also performed.

Blood samples were obtained for estimation of BNP before starting the first cycle of chemotherapy and after completion of the sixth cycle. Blood was withdrawn for assessment of H-FABP within 24 hours of administration of doxorubicin in the first cycle of CHOP. H-FABP was measured utilizing human cardiac fatty acid-binding protein ELISA test kit, Oxis research catalog number: 11230, Oxis International Inc., Foster City, CA, USA.

Re-evaluation of ECHO findings regarding the left ventricular ejection fraction and parameters of diastolic function was carried out after the end of the sixth cycle of chemotherapy.

Materials and Methods

Our study was conducted on 40 patients with non-Hodgkin’s lymphoma who received 6 cycles of an anthracycline containing regimen (CHOP) with the following doses:[1]

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose</th>
<th>Route</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophosphamide</td>
<td>750 mg/m²</td>
<td>IV Day 1</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>50 mg/m²</td>
<td>IV Day 1</td>
<td></td>
</tr>
<tr>
<td>Vincristine</td>
<td>1.4 mg/m²</td>
<td>IV Day 1</td>
<td></td>
</tr>
<tr>
<td>Prednisone</td>
<td>100 mg/d</td>
<td>PO Days 1-5</td>
<td></td>
</tr>
</tbody>
</table>

This cycle was repeated every 21 days.

Results

Patients’ data are summarized in Table 1. The mean age of our patients at presentation was 36.2±10.02 years. Fifty-five percent were males, while 45% were females. All patients had normal ECG findings with no clinical evi...
Evidence of heart failure prior to chemotherapy.

After completing the first cycle of CHOP, plasma levels of H-FABP were found to be elevated in 10 patients. After completion of the sixth cycle of chemotherapy, 8 of the 10 patients with elevated H-FABP showed a significant reduction in the left ventricular ejection fraction to levels below 50% and the reduction in EF showed a significant correlation with H-FABP levels (Figure 1). Six of them also showed evidence of diastolic dysfunction in the form of impaired relaxation and an E/A ratio below 1. Two patients of those with post-chemotherapy ejection fraction values less than 50% had clinical manifestations of heart failure. Fifteen of our patients (37.5%) showed evidence of diastolic dysfunction after 6 cycles of chemotherapy but 3 of them already had E/A ratios below 1 when evaluated before starting chemotherapy. We divided our patients into two groups according to echocardiographic evidence of left ventricular systolic dysfunction after the sixth cycle of chemotherapy (Table 2): Group I; patients who had left ventricular ejection fraction levels below 50%; Group II; patients who had no echocardiographic evidence of heart failure.

On comparing the mean plasma BNP values in both groups, we found that prior to chemotherapy, they did not show a significant difference (mean value group I 70.28±9.45, group II 75.62±11.84). After six cycles of chemotherapy, both groups differed significantly as regards plasma BNP, with the mean value in group I being 70.4±7.92 and in group II being 260±18.5 (p<0.001). There was also a significant elevation of BNP values in group II patients after chemotherapy and this elevation correlated significantly with the reduced ejection fraction in these patients (Figure 2). There were no patients in whom clinical cardiac failure occurred without an associated rise in the BNP level above the threshold value. From the 10 patients who exhibited elevated H-FABP levels after the first chemotherapy cycle, 8 also showed high BNP levels at the end of the sixth cycle and both biochemical markers correlated positively with each other (Figure 3).

**Discussion**

Cardiotoxicity leading to congestive heart failure is a well-known complication of anthracyclines. Biochemical methods to assess and monitor cardiac function after anthracycline administration, if informative would be of utmost value.12 We examined the diagnostic role of human heart-type fatty acid binding protein and brain natriuretic peptide to predict the impairment of left ventricular function in NHL patients treated by CHOP. We also studied the correlations between the plasma concentrations of these biomarkers and the functional alterations associated with doxorubicin-induced myocardial damage. A significant correlation between the left ventricular ejection fraction after 6 cycles of CHOP and plasma levels of both H-FABP and BNP was found in our patients. Pichon and colleagues stated that an infra-clinical cardiotoxicity of anthracyclines as defined by BNP elevation is frequent but reversible and that patients who developed heart failure showed a continuous BNP increase and concentrations over 100 ng/mL.16 On the other hand, Daugaard and colleagues concluded that in spite of correlations between peptide concentrations and reduced ejection fraction values, neither baseline levels nor serial measurements can safely substitute EF monitoring in patients undergoing anthracycline therapy.10 Brain natriuretic peptide (BNP) was originally discovered in the porcine brain but was subsequently found to be predominantly a cardiac hormone.17 Unlike atrial

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**Table 1. Biochemical markers and ECHO findings in our patients.**

<table>
<thead>
<tr>
<th></th>
<th>N.</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>40</td>
<td>40</td>
<td>14</td>
<td>36.2±10.02</td>
</tr>
<tr>
<td>BNP before chemotherapy (pg/mL)</td>
<td>40</td>
<td>50</td>
<td>89</td>
<td>71.35±10.04</td>
</tr>
<tr>
<td>BNP after chemotherapy (pg/mL)</td>
<td>40</td>
<td>50</td>
<td>280</td>
<td>108.32±77.66</td>
</tr>
<tr>
<td>H-FABP (ng/mL)</td>
<td>40</td>
<td>8</td>
<td>30</td>
<td>17.25±6.44</td>
</tr>
<tr>
<td>EF before chemotherapy (%)</td>
<td>40</td>
<td>58</td>
<td>72</td>
<td>65.57±4.04</td>
</tr>
<tr>
<td>EF after chemotherapy (%)</td>
<td>40</td>
<td>40</td>
<td>70</td>
<td>61.4±9.79</td>
</tr>
</tbody>
</table>

**Table 2. Comparison between group I and II patients as regards BNP and H-FABP.**

<table>
<thead>
<tr>
<th></th>
<th>N.</th>
<th>Mean</th>
<th>T</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td>32</td>
<td>-1.185</td>
<td>0.265</td>
</tr>
<tr>
<td>BNP before chemotherapy (pg/mL)</td>
<td></td>
<td>32</td>
<td>-1.185</td>
<td>0.265</td>
</tr>
<tr>
<td>BNP after chemotherapy (pg/mL)</td>
<td></td>
<td>32</td>
<td>-28.048</td>
<td>0.000*</td>
</tr>
<tr>
<td>H-FABP (ng/mL)</td>
<td></td>
<td>32</td>
<td>-13.103</td>
<td>0.000*</td>
</tr>
<tr>
<td>EF before chemotherapy (%)</td>
<td></td>
<td>32</td>
<td>0.903</td>
<td>0.384</td>
</tr>
<tr>
<td>EF after chemotherapy (%)</td>
<td></td>
<td>32</td>
<td>15.47</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

* p Significant. GI: Patients with post chemotherapy EF above 50%. GII: Patients with post-chemotherapy EF below 50%.

**Figure 1. Correlation between ejection fraction after 6 cycles of CHOP and H-FABP.**
natriuretic peptide (ANP), which is secreted by the atria in response to increased atrial pressure, BNP is derived chiefly from the cardiac ventricles in response to ventricular stresses. The plasma levels of both peptides are inversely correlated with measures of cardiac function and recent studies have shown BNP to be a more sensitive marker of cardiac impairment than ANP. Raised plasma BNP levels have previously been shown to herald the clinical picture of cardiac failure by days to weeks. Various guidelines based on changes in systolic and diastolic left ventricular function determined either by ECHO or by radionuclide ventriculography (RVG) have been proposed for monitoring patients receiving anthracycline therapy. The advantages of ECHO over RVG are better availability, lower costs and lack of exposure to ionizing radiation. Although H-FABP is viewed as a marker of myocyte injury and BNP is considered indicative of ventricular strain, the finding that both markers correlated with each other in our patients shows that some degree of myocyte injury is associated with increased ventricular load. It is well-known that myocardial structure is also altered in congestive heart failure (CHF). Non-contiguous areas of myocardial cell death and foci of replacement fibrosis are typical morphological changes in advanced CHF. Therefore, cytosolic proteins may be released into the circulation through leakage due to increased permeability of the membranes of injured myocytes. Thus, the present study highlights the practical importance of measuring some biochemical markers such as plasma H-FABP and BNP to monitor left ventricular dysfunction in patients receiving anthracycline therapy as an early indication of subclinical cardiotoxicity.

References


Palliative splenic irradiation in primary and post PV/ET myelofibrosis: outcomes and toxicity of three radiation schedules

Mario Federico,1 Guido Pagnucco,2 Antonio Russo,2 Giovanni Cardinali,2 Patrizia Guerrieri,3 Francesco Sciumè,4 Catherine Symonds,4 Letizia Cito,4 Sergio Siragusa,4 Nicola Gabbia,5 Roberto Lagalla,1 Massimo Minieri,1 Antonio Giordano,1 Paolo Montemaggi2
1U.O. Radioterapia, DiBiMeL, Università degli Studi di Palermo, Italy
2U.O. Radioterapia, ARNAS Civico, Dipartimento Oncologico M. Ascoli, Palermo, Italy
3U.O. Ematologia, ARNAS Civico, Dipartimento Oncologico M. Ascoli, Palermo, Italy
4U.O. Ematologia, Università degli Studi di Palermo, Italy
5U.O. Oncologia Medica, Dipartimento di Chirurgia e Oncologia, Università degli Studi di Palermo, Italy
6Barro Health Research Organization, Temple University, Philadelphia, USA
7CROM, Centro Ricerche Oncologiche, Mercogliano, Italy

Abstract

Splenectomy and splenic irradiation (SI) are the sole treatment modalities to control drug resistant splenomegaly in patients with myelofibrosis (MF). SI has been used in poor surgical candidates but optimal total dose and fractionation are unclear. We retrospectively reviewed 14 MF patients with symptomatic splenomegaly. Patients received a median of 10 fractions in two weeks. Fraction size ranged from 0.2-1.4 Gy, and total dose varied from 2-10.8 Gy per RT course. Overall results indicate that 81.8% of radiation courses achieved a significant spleen reduction. Splenic pain relief and gastrointestinal symptoms reduction were obtained in 94% and 91% of courses, respectively. Severe cytopenias occurred in 13% of radiation courses. Furthermore patients were divided in three groups according to the radiation dose they received: 6 patients in the low-dose group (LDG) received a normalized dose of 1.67 Gy; 4 patients in the intermediate-dose group (IDG) received a normalized dose of 4.37 Gy; the remaining 4 patients in the high-dose group (HDG) received a normalized dose of 9.2 Gy. Subgroup analysis showed that if no differences in terms of treatment efficacy were seen among dose groups, hematologic toxicity rates distributed differently. Severe cytopenias occurred in 50% of courses in the HDG, and in the 14.3% and in 0% of the IDG and LDG, respectively. Spleen reduction and pain relief lasted for a median of 5.5 months in all groups. Due to the efficacy and tolerability of the low-dose irradiation 4 patients from the LDG and IDG were retreated and received on the whole 12 RT courses. Multiple retreatments did not show decremental trends in terms of rates of response to radiation nor in terms of duration of clinical response. Moreover, retreatment courses did not cause an increased rate of adverse effects and none of the retreated patients experienced severe hematologic toxicities. The average of clinical benefit in retreated patients was much longer (21 months, range 4-10) than patients who were not retreated (3.75 months, range 3-6).

Introduction

Primary myelofibrosis1 (PM) is a Philadelphia negative chronic myeloid disorder (CMD) currently classified with polycythemia vera (PV) and essential thrombocytelmia (ET) as a chronic myeloproliferative disease2 (MPDs). PM is a rare disease mainly affecting older people3 with a median survival of 3.5-5 years.4 The pathogenetic mechanism is not clearly understood but probably relates to a clonal stem-cell disorder that leads to ineffective erythropoiesis, dysplastic megakaryocyte hyperplasia and an increased ratio of immature to total granulocytes.5 These findings are characteristically accompanied by reactive bone marrow (BM) fibrosis that develops and is mediated by megakaryocyte-derived fibrogenic cytokines.6

Collagen fibrosis, presumably along with many other factors, interferes with normal hematopoietic processes, ultimately leading to erythroid hypoplasia.7 Due to BM fibrosis, in MF patients as well as those with post ET/PMF, an extramedullary hematopoietic process starts in the spleen or in multiple organs as an attempt to override BM failure, often leading to the development of splenomegaly or hepato-splenomegaly. Moreover, splenomegaly exacerbates cytopenias through the sequestration and destruction of hematopoietic elements.7

Progressive high-grade splenomegaly occurs in the majority of MF patients. Unfortunately the standard current pharmacological therapeutic options, due to their short periods of response, fail to control organomegaly and organomegaly-associated symptoms (abdominal pain and early satiety, weight loss, portal hypertension and profound fatigue), which account for much of the patient’s discomfort. Also even though a new generation of “target drugs” are currently under intense investigation with some encouraging results, splenomegaly control still remains a crucial step for patients’ quality of life improvement.

To date, splenectomy or splenic irradiation (SI) are the sole treatment modalities to control drug resistant splenomegaly in MF patients. When technically achievable splenectomy is currently the preferred treatment modality for MF based upon good, long-lasting outcome in term of organomegaly-related symptom palliation.8,9 Unfortunately, it is consistently associated with a significant rate of mortality as well as intra- and peri-operative complications.10,11 SI, instead, has been generally preferred in patients not undergoing surgery due to a poor general status or decline and allows for a good but transitory splenomegaly palliation. In fact, the major shortcoming of radiation is that its palliative effect on splenomegaly generally does not last longer than six months.

There is a general agreement that emerges from the literature to use RT at dose levels lower than in other hematologic malignancies; however, few studies have taken a retrospective look at SI.11 The indication for SI is still controversial12 and there is not a precise unequivocal definition of the optimal total dose and fractionation, mainly due to the limited number of patients included in existing studies and the wide range of radiation schedules adopted. Moreover, it is unclear if re-irradiation of MF patients is a safe strategy to extend the overall time of clinical benefit that a single SI course allows. Here we aim to assess outcomes and complication rates of
splenic irradiation in three cohorts of patients treated with different “low-dose” irradiation schedules.

**Patients and Methods**

After approval from our institutional research review committee, we retrospectively reviewed data concerning 15 patients (10 male, 5 female, median age at diagnosis 61 years, median age at first irradiation 67 years), 11 with a histologically proven diagnosis of PM and 4 with a post ET-MF complaining of a high-grade symptomatic splenomegaly that were consecutively referred to our institution from 1997-2007 (Table 1). All patients had a drug resistant splenomegaly and lacked any further treatment options. Before being admitted to irradiation, patients had previously been judged unfit for surgery due to their general status or had refused splenectomy. Fourteen out of 15 underwent splenic irradiation and one was excluded due to pre-existing advanced heart failure (patient 14). In the 14 irradiated patients the first course of radiation occurred at a median of 58 months from the diagnosis of MF. All 14 treated patients had a severe resistant splenomegaly and lack of any further treatment options. Before being admitted to irradiation diagnosis score treatments at time transfusion irradiation WBC irradiation PLT

**Table 1. Patients’ characteristics at the time of first irradiation.**

<table>
<thead>
<tr>
<th>Patient N.</th>
<th>Age at irradiation and sex</th>
<th>Interval diagnosis irradiation (intent to treat)</th>
<th>Dupriez score</th>
<th>Previous treatments</th>
<th>Symptoms at time of radiation</th>
<th>RBC transfusion U/month</th>
<th>Pre-irradiation WBC ×10^9/L</th>
<th>Pre-irradiation PLT ×10^9/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>63 F</td>
<td>14 y.</td>
<td>HR</td>
<td>HU, Th, S, P</td>
<td>2U</td>
<td>39.9</td>
<td>232</td>
<td></td>
</tr>
<tr>
<td>2*</td>
<td>65 F</td>
<td>16 y.</td>
<td>IR</td>
<td>HU</td>
<td>4U</td>
<td>6.48</td>
<td>381</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>62 F</td>
<td>3 y.</td>
<td>LR</td>
<td>HU, MPH, S, P</td>
<td>2U</td>
<td>9.55</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>4*</td>
<td>75 F</td>
<td>8 y.</td>
<td>IR</td>
<td>HU, S, P, CS</td>
<td>2U</td>
<td>8.36</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>67 M</td>
<td>4 y.</td>
<td>IR</td>
<td>HU</td>
<td>2U</td>
<td>3.7</td>
<td>210</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>67 M</td>
<td>8 y.</td>
<td>IR</td>
<td>HU, ARA-C</td>
<td>NT</td>
<td>38.1</td>
<td>190</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>77 M</td>
<td>8 y.</td>
<td>IR</td>
<td>HU, 6-MP, S, P</td>
<td>2U</td>
<td>10.3</td>
<td>423</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>87 M</td>
<td>1 y.</td>
<td>IR</td>
<td>HU</td>
<td>2U</td>
<td>2.63</td>
<td>119</td>
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</tr>
<tr>
<td>9</td>
<td>45 M</td>
<td>4 y.</td>
<td>IR</td>
<td>HU</td>
<td>NT</td>
<td>29.6</td>
<td>307</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>67 M</td>
<td>1 y.</td>
<td>IR</td>
<td>HU</td>
<td>2U</td>
<td>4.9</td>
<td>121</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>70 M</td>
<td>2 y.</td>
<td>IR</td>
<td>HU</td>
<td>2U</td>
<td>88</td>
<td>143</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>58 M</td>
<td>7 y.</td>
<td>IR</td>
<td>HU, Th, S, P, night sweating</td>
<td>2U</td>
<td>10.89</td>
<td>329</td>
<td></td>
</tr>
<tr>
<td>13*</td>
<td>65 M</td>
<td>2 y.</td>
<td>IR</td>
<td>HU, 6-MP, Th</td>
<td>2U</td>
<td>8</td>
<td>673</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>76 M</td>
<td>4 y.</td>
<td>IR</td>
<td>HU</td>
<td>4U</td>
<td>1.04</td>
<td>67</td>
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</tr>
<tr>
<td>15</td>
<td>55 F</td>
<td>4 y.</td>
<td>IR</td>
<td>HU, Th</td>
<td>2U</td>
<td>42.51</td>
<td>143</td>
<td></td>
</tr>
</tbody>
</table>

S: Splenomegaly; P: splenic pain; CS: constitutional symptom; HU: Hydroxyurea; Th: Thalidomide; 6-MP: 6-mercaptopurine; ARA-C: Arabinosylcytosine; MPH: Melphalan; B: Busulphan; HR: High-risk; IR: Intermediate-risk; LR: Low-risk; NT: Not transfused; *Post ET - MF.

Since our institutional standards of radiation for MF have changed during the past ten years, patients received different total doses and dose per fraction. To compare the various RT treatments we used the Normalized Tumor Dose (NTD), defined as the total dose delivered in 2 Gy fractions that corresponds to a particular biologically effective dose level and is calculated according to the formula:

\[
NTD_n = nd \frac{1 + \frac{d}{\alpha}}{1 + \frac{2}{\beta}}
\]

where n is the number of RT fractions and d the fraction size in Gy. The \(\alpha/\beta\) value of the Linear Quadratic Model was empirically fixed to 10 as for early responding tissues. By standardizing the delivered dose of all 22 administered treatments into a 2 Gy isoeffective treatment, we were able to make a correct radiobiological comparison among different RT schedules. On the basis of the NTD values, patients were divided into three different groups but it should be underlined that RT schedules were not chosen on the basis of patients’ clinical parameters but rather were dependent on the progressive modification of our institutional treatment philosophy.

The initial patients, who had received a total dose of 10 Gy with a dose per fraction in the order of 1 Gy, were designated as our high-dose group (HDG). Patients who had received our current standard of treatment (0.2 Gy fraction up to a total dose of 2 Gy in 10 fractions) were designated as our low-dose group (LDG).
Whereas the intermediate-dose group (IDG) reflected the transition or better our “dose finding effort” toward lower doses with the aim to reduce treatment related toxicities (Supplementary Tables). The IDG encompassed patients who had received a wide range of treatment with radiobiological characteristics, which, in some cases, may partially overlap with the LDG. However, we decided to aggregate our patients in this way in order to obtain homogeneity in the low- and in the high-dose groups.

 Patients in which three or more of the following criteria were present were considered responsive to treatment: subjective absence of MF-related gastrointestinal symptoms (bulky effect), absence of splenic pain, consistent reduction of the spleen volume (not less than 50% of the initial size) assessed by clinical examination (according to the formula: spleen volume $= \frac{4}{3} \pi (1^\text{st} \text{diameter} \times 2^\text{nd} \text{diameter} \times 3^\text{rd} \text{diameter})$) and, finally, reduction of the RBC transfusion units required per month.

To evaluate toxicity and response to treatment, patients had undergone clinical examination and blood cell count twice a week during the period of irradiation and for the following two weeks. If no toxicity occurred, blood tests were scheduled weekly for the following month. The evaluation of the spleen reduction was carried out 20 days after patients had completed radiation.

Treatment related toxicity was limited to myelosuppression and was measured on the basis of RTOG acute toxicity scoring criteria. An RT course after which a post-radiation WBC count $\leq 1\times10^9/L$ and/or PLTs count $\leq 20\times10^9/L$ acute cytopenia had developed was considered too toxic. RBC count was considered to be grade 4 (WBC count $\leq 1\times10^9/L$ acute cytopenia had developed was considered too toxic. RBC count was considered to be grade 4 (WBC count $\leq 1\times10^9/L$ and/or PLTs count $\leq 20\times10^9/L$ acute cytopenia had developed was considered too toxic. RBC count was considered to be grade 4 acute toxicity). Two of the retreated patients (patients #9 and #8) experienced severe acute complications. One (patient #8) appeared to have been rescued from cytopenia but three months later developed a leukemic transformation that led to death. The second patient (patient #9), complained of a massive splenomegaly, did not respond to SI and underwent splenectomy 12 months after RT. One month after splenectomy the patient died as a result of sepsis.

The median time of symptom relief after a single RT course was 5.5 months and no differences were found among dosage groups. According to the patients’ general conditions, the cumulative RT dose delivered and the rate of spleen shrinkage in response to previous irradiation, retreatment after splenic relapse was considered in 4 patients. The 4 retreated patients received on the whole 12 RT courses and one patient received 4 courses without any acute toxicity. Two of the retreated patients belonged to the LDG and the remaining 2 to the IDG. However, it is important to note that of the patients retreated from the IDG, one

### Table 2. Splenic irradiation results (by NTD group).

<table>
<thead>
<tr>
<th></th>
<th>Number of patients</th>
<th>Median dose delivered</th>
<th>Median Dose per fraction</th>
<th>NTD10 m sd</th>
<th>% of courses with reduction in spleen size</th>
<th>% of courses with pain relief</th>
<th>Response</th>
<th>Median duration of response in months</th>
<th>Hematologic toxicity Grade 4 RTOG</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDR</td>
<td>3</td>
<td>3</td>
<td>200 cGy (2-4 Gy range)</td>
<td>11</td>
<td>20 cGy</td>
<td>1.67 Gy</td>
<td>0.60</td>
<td>91%</td>
<td>100%</td>
</tr>
<tr>
<td>HDR</td>
<td>4</td>
<td>0</td>
<td>1000 cGy (900-1000 cGy)</td>
<td>4</td>
<td>110 cGy</td>
<td>9.20 Gy</td>
<td>0.46</td>
<td>75%</td>
<td>100%</td>
</tr>
<tr>
<td>IDR</td>
<td>3</td>
<td>1</td>
<td>500 cGy Range</td>
<td>7</td>
<td>50 cGy</td>
<td>4.37 Gy</td>
<td>1.89</td>
<td>76.5%</td>
<td>86%</td>
</tr>
</tbody>
</table>

Low-dose group (LDG); high-dose group (HDG); intermediate-dose group (IDG); median (m) standard deviation (sd).
received treatments of 0.3 up to 3 Gy in 10 fractions total, which could be considered radiobiologically partially overlapping with the treatment dosages of the LDG.

In comparison to the first irradiation, multiple retreatments did not show decremental trends in terms of rates of response to radiation nor in terms of duration of clinical response. Even in the case of one patient, who received 4 RT courses, there was no change in the duration of symptoms' palliation. Moreover, after retreatment courses we did not observe an increased rate of adverse effects and none of the retreated patients experienced severe hematologic toxicities. The average time of clinical benefit (Supplementary Figure 2) in retreated patients was very much longer (21 months, range 44-10) than patients who were not retreated (3.75 months, range 3-6).

**Discussion**

Splenomegaly rapidly occurs in all MF patients and is one of the causes of major discomfort. Curative treatments are to-date still limited in MF. Allogeneic bone marrow transplantation (allo-BMT) has shown promising results in younger patients but its role in elderly patients is controversial. In particular, several studies suggest that in individuals older than 45 the treatment's risk-related mortality outweighs the benefits.\(^\text{27}\) On the contrary, other studies more recently explored the use of allo-BMT also in patients older than 60 with some interesting results.\(^\text{30}\) Currently, BMT in the elderly is still a matter of debate since the number of patients accrued in clinical trials is limited and the follow-up time short. Since MF remains a disease of the elderly, standard and palliative treatments to manage cytopenias and massive organomegaly still retain a relevant role in a consistent proportion of patients.

Splenomegaly can be effectively controlled by conventional cytotoxic chemotherapy\(^\text{24}\) until patients become drug resistant. More recently atiangiogenic drugs and target drugs are expected to offer a new chance of treatment for all patients. In particular a new class of molecules designed to inhibit Jak have been tested in different phase II trials with positive results.\(^\text{25}\) Jak inhibitors have shown a significant activity on splenomegaly but there is no reason to think that, along with their use, also resistant patients will be selected.

After massive splenomegaly is established, splenectomy is considered the principle palliative measure because it offers a lengthy relief of symptoms. Unfortunately splenectomy is weighted by significant morbidity and mortality rates. The two largest single institution series from Barosi\(^\text{26}\) and Tefferi\(^\text{27}\) reported a mortality rate of 8.4% and 9%, respectively, with the latter increasing to 26% when the three-month post-splenectomy period was considered, and a morbidity rate of 39.3% and 31%, respectively. After splenectomy, up to 25% of patients may experience accelerated hepatomegaly and extreme thrombocytosis.\(^\text{28}\) Moreover, splenectomy has also been correlated to a significantly higher incidence of blast transformation.

A large Italian study demonstrated a crude transformation rate in splenectomized patients of 26.4% in comparison to 11.9% in non-splenectomized patients with the cumulative actuarial transformation rate of 55% in splenectomized vs. 27% in non-splenectomized patients at 12 years after diagnosis. The overall risk of blast transformation was therefore 2.61 times higher among splenectomized patients.\(^\text{29}\) In conclusion, despite the impact on symptoms, no overall survival benefit has been demonstrated after splenectomy\(^\text{23}\) on the contrary, this procedure is associated with a substantial risk of operative mortality, early and late morbidity and is contraindicated in patients with thrombocytosis. Furthermore, splenectomy has been shown to be a predictor of treatment failure in case of allo-BMT.\(^\text{30}\)

Alternative treatments to manage splenomegaly, with lower morbidity and mortality rates, would offer a significant improvement in the clinical management of MF patients.

Radiotherapy has been used in selected situations to control extramedullary hematopoiesis, as in spinal localizations,\(^\text{26}\) in pulmonary hypertensin\(^\text{31}\) or in symptomatic hepatomegaly\(^\text{32}\) with promising results. However, its role in splenic palliation remains controversial because of the lack of robust data (Table 3). It has been shown that splenic irradiation can be very effective in reducing spleen size and splenic pain with response rates comparable to splenectomy.\(^\text{33}\) The major shortcoming of radiotherapy is the reliance on its transient effect that normally does not exceed six months.

As an alternative to splenectomy, SI has been considered in poor surgical candidates or in patients who declined surgery. In these patients, that generally are in a worse condition compared to those that undergo surgery, palliative splenic irradiation has shown mortality rates that are comparable to splenectomy.\(^\text{34}\) On the other hand, a high rate of severe life-threatening cytopenias has been reported in patients that underwent splenic irradiation, ranging from 32% (1650 courses) of the Mayo Clinic series\(^\text{35}\) where lower doses of RT were used (median dose per course 2.77 Gy) to 35% (6/17 courses) of a French series\(^\text{36}\) where a more aggressive treatment was delivered (median dose per course 9.8 Gy).

Although a general trend in favor of low doses is emerging in the literature, the wide variability of total radiation doses, the different number of fractions, as well as the different schedule of irradiation reported, makes it difficult to define a standard of treatment.\(^\text{37}\)

In order to be able to make dose-effect comparisons, the major drawback of some of the published series is that the total dose and the fractionation scheme seem not to be decided up-front from the treatment but modified during the irradiation on the basis of the single patient response with a consequent high variability in the total dose, fractionation and overall treatment time. Some authors\(^\text{38}\) used the common 5 daily fractions per week schedule but increased the fraction size during the radiation course (from 0.4-0.5 Gy/fraction in the first week of treatment, up to 0.8-1 Gy/fraction during the following weeks) until the palliative effect or toxicity is reached. Other authors\(^\text{39}\) give radiation 2-3 times per week with an altered time factor. Both such approaches can be empirically effective but generate data that are difficult to compare with the common radiotherapy algorithms that are based on larger fraction sizes (around 2 Gy) and with a time of inter-course sub-lethal DNA damage repair of 24 hours between fractions. Given that it is hard to make radiotherapeutic comparisons among some published series, it is clear that, still now, the most critical issue regarding a rational use of RT is the definition of an optimal total dose and fractionation.

The leading idea of our approach to SI has been to adopt a relative long fractionation schedule of 10 fractions in two weeks independent of the total dose delivered with the intent to generate comparable results, also in case of treatments differing in total dose and dose per fraction. This approach should also minimize the incidence rate of post-attinic severe cytopenias and favor a rapid recovery of early blood precursors from RT. In fact, since a strong dose-sparing effect of fractionation on bone marrow precursors\(^\text{40,41}\) has been proven, we believed that it would be meaningful to also apply this concept to extramedullary hematopoiesis sites. Therefore, we decided to utilize a long RT schedule (median 10 fractions) even when it could appear unjustified to do so due to the minimal total dose delivered.

Regarding the total dose, at the beginning of our experience, we adopted an aggressive RT regimen (1 Gy per fraction up to a total dose of 10 Gy) but we observed a high incidence of severe side effects. This raised the concern that the same stem clonal disorder that underlies MF could make hematopoietic precursors more sensitive to radiation. In order to reduce the incidence of acute cytopenias we progressively reduced total RT doses until we established our actual standard of care (0.2 Gy per fraction up to 2 Gy total dose).

Our findings show that extremely low-dose treatments are isoeffective as compared to higher dose regimens in effectively reducing
splenomegaly. Unfortunately, we cannot explain the functionality of low-dose treatment regimens in being so effective as compared to high-dose treatments; however, these findings are in concordance with the hypothesis of low-dose hypersensitivity.36 The suggestive issue of radiobiology has been intensely investigated in vitro and postulates a hypersensivity state of cells when irradiated at low doses (<0.4-0.5 Gy). Recently, there have been several indirect confirmations of this theory in clinical studies, linking low-dose hypersensitivity to tumor regression as well as to the occurrence of adverse effects, at dose levels under the threshold generally accepted for toxicity or tumor control.

Since in our series, as well as in others reported,27-29 there is an inherent discrepancy due to variability in total dose delivered, fraction number, and fraction size, to be able to correctly compare different treatments we used the NTD formula, a radiobiological tool commonly used in the clinic to evaluate the biological effectiveness of modified RT fractionations. The overall NTD of all 22 RT courses in our series is 2.59 Gy, a value comparable with the median NTD estimated from the Mayo series (3.16 Gy). Interestingly our patients seem to have a lower overall incidence rate of grade 4 RTOG (13.6% of courses vs. 32%). This discrepancy is somehow difficult to be explained since there are just slight differences in the normalized radiation dose that patients of the two groups received. Even a slighter difference in terms of patient characteristics can be found between the Mayo Clinic series and ours (median age at the time of the first irradiation 65 vs. 67 years; time intercurring between diagnosis and irradiation 44 vs. 58 months, respectively). A possible explanation could be that in the definition of toxicity criteria, differing from the Mayo report, we did not consider hemoglobin levels since the majority of our patients were transfused from a long time before receiving radiotherapy. Another possible explanation could rely on the medical treatment that patients received before undergoing radiation: in fact, it is interesting to note that the only 2 patients in both series that received melphalan as medical treatment before radiation later experienced severe post-attinic cytopenias.

To compare outcomes after different radiation doses we stratified our patients into three groups according to the NTD value they received. A possible explanation could be that in the definition of toxicity criteria, differing from the Mayo report, we did not consider hemoglobin levels since the majority of our patients were transfused from a long time before receiving radiotherapy. Another possible explanation could rely on the medical treatment that patients received before undergoing radiation: in fact, it is interesting to note that the only 2 patients in both series that received melphalan as medical treatment before radiation later experienced severe post-attinic cytopenias.

<table>
<thead>
<tr>
<th>Author</th>
<th>Number of patients</th>
<th>Median dose delivered</th>
<th># of RT courses</th>
<th>Median dose per fraction</th>
<th>Estimated NTD10 Median</th>
<th>% of courses with reduction in spleen size</th>
<th>% of courses with pain relief</th>
<th>Median duration of response (in months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elliot</td>
<td>18</td>
<td>5</td>
<td>277.5 cGy range (30-1365 cGy)</td>
<td>50</td>
<td>50 cGy</td>
<td>3,162 cGy</td>
<td>2,784</td>
<td>94%</td>
</tr>
<tr>
<td>Greenberger</td>
<td>13</td>
<td>1</td>
<td>650 cGy range (40-1720 cGy)</td>
<td>21</td>
<td>57.14 cGy</td>
<td>5,807 cGy</td>
<td>3,204</td>
<td>95%</td>
</tr>
<tr>
<td>Parmentier</td>
<td>5</td>
<td>4</td>
<td>690 cGy range (100-290 cGy)</td>
<td>12</td>
<td>25 cGy range (12,5-75)</td>
<td>5,845 cGy</td>
<td>6,451</td>
<td>92%</td>
</tr>
<tr>
<td>Wagner</td>
<td>6</td>
<td>0</td>
<td>NA From 200-450 cGy in 25-30 fraction 3 times per week</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>80%</td>
</tr>
<tr>
<td>Bonadelallah</td>
<td>15</td>
<td>0</td>
<td>900 cGy range (40-3050 cGy)</td>
<td>17</td>
<td>Daily fr. 40-100 cGy median duration 22 days</td>
<td>NA</td>
<td>NA</td>
<td>81%</td>
</tr>
<tr>
<td>Mc Farland</td>
<td>4</td>
<td>2</td>
<td>range 300-600 cGy</td>
<td>13</td>
<td>Irradiation twice wc: 1stw50 cGy 2ndw35 cGy 3rdw100cGy</td>
<td>NA</td>
<td>NA</td>
<td>92%</td>
</tr>
<tr>
<td>Present study</td>
<td>LDG 3</td>
<td>3</td>
<td>200 cGy range (200-400 cGy)</td>
<td>11</td>
<td>26 cGy</td>
<td>1.67 cGy</td>
<td>0.603</td>
<td>91%</td>
</tr>
<tr>
<td></td>
<td>HDG 4</td>
<td>0</td>
<td>1000 cGy range (880-1080 cGy)</td>
<td>4</td>
<td>110 cGy</td>
<td>9.205 cGy</td>
<td>0.465</td>
<td>75%</td>
</tr>
<tr>
<td></td>
<td>IDG 3</td>
<td>1</td>
<td>500 cGy range (300-800 cGy)</td>
<td>7</td>
<td>50 cGy</td>
<td>4,375 cGy</td>
<td>1.892</td>
<td>76.5%</td>
</tr>
</tbody>
</table>

NA: not assessable.
scheme since it is not associated to grade 4 hematologic toxicities. In our patients, independently from the dose, radiotherapy was very effective in reducing massive splenomegaly, but did not resolve completely the spleen enlargement (Online Supplementary Tables). It is possible to argue that, since we found a safe RT schedule, it would be meaningful to prolong the radiation treatment until a complete splenomegaly remission. On the contrary, we decided to maintain a conservative approach and to stop the treatment once the planned final dose was achieved. Two main considerations led to our decision: first of all the fact that the palliative effect of radiotherapy seems to last no longer than six months independently from the dose delivered.

We were concerned that reducing the spleen size until normalization could result in a small increase of the time free from symptoms at the cost of a probably higher incidence of severe cytopenias. Secondly, since the aim of our treatment was strictly palliative, we considered it meaningful, once symptom relief was achieved, to stop the treatment with the intent to minimize the patient’s absorbed dose per course of RT in order to potentially be able to repeat the treatment in the future.

In fact, because of the low incidence of mild adverse effects in the LDG (and in the lower dose burden of the IDG) we were able to repeat the irradiation several times thus prolonging the clinical benefit much more than expected.

Four patients safely underwent 12 RT courses with no occurrence of grade 4 RTOG hematologic toxicity. All the retreated patients belonged to the low- or intermediate-risk group. In these patients the intensity and the persistence of splenic response to irradiation did not change under multiple retreatment courses. However, retreatment increased the average time of symptoms relief four fold longer than in un-retreated patients (21 vs. 5.75 months). It could be argued that a possible bias in our work is that all the post ET-MF patients were allocated in the LDG or in the inferior burden of other IDG but this fact could not modify the consistency of the presented data; especially because just one patient with post ET-MF has been retreated so far. We propose that the results regarding the average time of clinical benefit in retreated patients can be considered substantially valid for primary MF patients. Furthermore, it deserves to be mentioned that, in comparison to MF patients, a shorter interval free from symptoms has been reported after SI in post-ET MF patients.

With all the limitations inherent in the small number of patients examined, we found that in our series Dupriez's score (calculated at the time of patient's referral to the radiotherapy department) is not predictive of response to palliative radiotherapy or occurrence of toxicity. We conclude that our actual standard of 2 Gy delivered in 10 fractions over two weeks has a NTD of 1.67 Gy, a value two to three-fold lower than other published series. This schedule of treatment has been shown to be extremely well tolerated and to date in our experience is not associated with severe hematologic toxicities. Such optimal treatment compliance encouraged repeating irradiation in responsive patients and this favored a drastic increase in the average time of clinical benefit.

References
24. Koch CA, Li CY, Mesa RA et al. Non hepatosplenic extramedullary hematopoiesis:
MicroRNAs: tiny players with a big role in the pathogenesis of leukemias and lymphomas

Francesca Fanini,1 Ivan Vannini,1 Muller Fabbri1,2
1Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori, Meldola, Italy;
2Department of Molecular Virology, Immunology, and Medical Genetics and Comprehensive Cancer Center, Ohio State University, Columbus, OH, USA

Abstract

MicroRNAs (miRNAs) are small non-coding RNAs (ncRNAs) with important regulatory functions. After an initial phase, aimed at identifying whether a deregulation in miRNA expression occurred between hematologic malignancies and their normal counterparts, currently an increasing number of studies are focusing on the functional significance of these aberrances. The identification of miRNA targeted genes has cast a new light on the role of these tiny ncRNAs in human cancerogenesis, providing a new rationale to the observed diagnostic, prognostic and therapeutic implications of miRNA aberrant expression in human hematologic malignancies.

Introduction

MicroRNAs (miRNAs) are a class of non-coding RNAs (ncRNAs) with regulatory functions, involved in a variety of biological processes.14 Initially transcribed by RNA polymerase II as long, capped and polyadenylated precursors (pri-miRNAs),15 miRNAs undergo a complex processing mechanism. First, a double-stranded RNA-specific ribonuclease called Drosha, in conjunction with its binding partner DGC8 (DiGeorge syndrome critical region gene 8, or Pasha) processes pri-miRNAs into hairpin RNAs of 70-100 nucleotides (nt) known as pre-miRNAs.16 By means of Exportin 5, pre-miRNA is translocated from the nucleus to the cytoplasm, where a ribonuclease complex, composed of a ribonuclease III (Dicer), and TRBP (HIV-1 transactivating response RNA binding protein) cleaves it in a 18-24 nt duplex. Finally, the duplex interacts with a large protein complex called RISC (RNA-induced silencing complex), which includes proteins of the Argonaute family (Ago1-4 in humans). One strand of the miRNA duplex remains stably associated with RISC and becomes the mature miRNA, which guides the RISC complex mainly (but not exclusively) to the 3′-UTR (3′-untranslated region) of the target miRNAs. The final outcome of the target miRNA is based upon the miRNA:mRNA degree of base pair complementarity. A perfect base pair match (occurring mainly in plants), leads to mRNA cleavage, whereas an imperfect complementarity (predominant in nematodes and mammals) results in translational silencing of the target, albeit also in case of imperfect base pairing a reduction of the target mRNA has been described.17 A further level of complexity in miRNA gene regulation has been recently provided by showing that miRNAs can also directly up-regulate the expression of a target gene by binding to its 3′-UTR.18

The development of high throughput methods to detect miRNA expression in human samples,19,20 has provided invaluable tools to investigate the role of these ncRNAs in several human pathologies. It is now generally accepted that miRNAs are aberrantly expressed in almost all human cancers (both solid and hematologic malignancies), with respect to the normal counterpart.13-15 This review will focus on the role of miRNAs in the pathogenesis of human leukemias and lymphomas, highlighting diagnostic, prognostic, and therapeutic implications of this class of ncRNAs. Table 1 summarizes the most frequently de-regulated miRNAs in hematologic malignancies.

miRNAs and leukemias

Leukemia is a malignant disorder of the blood or bone marrow characterized by an abnormal proliferation of blood cells. Based on the stage of differentiation of the malignant cells, leukemias can be divided into acute and chronic. Acute leukemias are characterized by the rapid increase of immature blood cells (blood cell progenitors or primitive stem cells with multilineage potential) and are the most common forms of leukemia in children, while chronic leukemias arise from more mature cells during differentiation and occur more often among adults. Additionally, the diseases are subdivided, according to the origin of the neoplastic cells, into lymphoid leukemias, concerning the lymphoid pathway (B and T cells) and myeloid leukemias, which involve the cells that normally go on to form red blood cells, granulocytes, monocytes and platelets. It was recently reported that the pattern of miRNA expression varies strongly during the differentiation of hematopoietic stem/progenitor cells, playing a critical role in events that result in hematologic disorders.24,17

miRNAs in chronic leukemias

Chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia, characterized by the slow accumulation in blood, bone marrow and lymphatic tissue of small, non-proliferating, mature B lymphocytes, which display typical surface markers such as CD19 and CD20 in addition to CDS.25

More than 50% of CLLs are characterized by hemizygous and/or homozygous deletion of the genomic region 13q14.25-28 Calin et al. showed that a cluster of miRNAs, namely miR-15a-16-1, is located at chromosome 13q14 and that these miRNAs, situated within a 30-kb region of loss in CLL, are both deleted or down-regulated in approximately 68% of CLL cases.21 Among the targets of miR-15a-16-1, was identified BCL2,22 an anti-apoptotic protein which is over-expressed in the majority of CLL B cells,22 and it is believed to mediate the anti-tumoral effect of these miRNAs. This finding is supported by the fact that restoration of miR15a-16 induces apoptosis in MEG-01 cell line, derived from acute megakaryocytic leukemia, pointing out a role for miR15a-16 as tumor-suppressor genes (TSGs) in CLL, and maybe in other malignancies in which this cluster is down-regulated or lost.22,23 Croce’s group first identified a unique microRNA signature associated with prognostic factors and disease progression in CLL,24 and described a germ-line mutation in pre-miR-16 sequence, which causes low levels of microRNA expression both in vitro and in vivo. This mutation was associated with deletion of the normal allele in leukemic cells of 2 CLL patients one of which with a family history of CLL and breast cancer.25 Interestingly, Raveche et al. described a point mutation in the 3′-DNA adjacent to...
miR-16-1 region, which lets down the expression of this miRNA in the CLL-prone New Zealand black mouse strain model, indicating that this miRNA functions as an oncosuppressor in CLL.26 By analyzing the result of miR15a/16-1 hyperexpression in MEG-01 cells and in CLL patients both at the transcriptome and at the proteome level, Calin et al. found that about 14% of all human genome is directly or indirectly affected by miR15a/16-1 cluster.26 Among the genes which are down-regulated by this cluster, there are both oncogenes (OGs) and TSGs, suggesting that even though the overall effect of miR15a/16-1 overexpression is anti-tumoral in CLL, it cannot be excluded that other pathways may be impacted and lead to an overall oncogenic outcome in other diseases. Further investigations are warranted to verify this hypothesis.

Another 2 miRNAs with a TGS function in CLL are miR-29b and miR-181b, which directly target TCL1 (T-cell leukemia/lymphoma 1A), an OG which co-activates the protein kinase AKT (v-akt murine thymoma viral oncogene homolog 1) and takes part in the regulation of many pathways involved in cell survival, proliferation and death.28 As evi...
miRNAs in acute leukemias

Acute lymphocytic leukemia

Acute lymphocytic leukemia (ALL) is one of the most common malignancies observed in the pediatric age and it represents about the 80% of cases of acute leukemia in children. It arises from the clonal proliferation of lymphoid progenitors in the bone marrow whose consequence is the occurrence of several different cytoplasmas in the peripheral blood associated with the appearance of peripheral blast cells. In 2007, Zanette et al. analyzed miRNA expression profiles in 7 ALL samples and 6 normal CD19+ samples in order to compare them. They first reported the involvement of miR-128b, miR-204, miR-218, miR-331 and miR-181b-1 in hematologic malignancies, with miR-128b as the most represented miRNA in ALL. In their analysis also the miR-17-92 cluster resulted up-regulated in all 7 samples. The nucleotide sequences and organization of this cluster is highly conserved in vertebrates, and its expression is tightly regulated during B-cell development. A role of miR-17-92 cluster to promote survival of early B-cell progenitors in a cell-autonomous manner, and control cell survival during the pro-B to pre-B transition was recently suggested by Ventura et al. They proposed that in human B-cell lymphomas miR-128 overexpression is induced at the pro-B stage and is associated with FLT3-ITD mutations. In a recent study, Fazi et al. found that expression levels of microRNA-181 family were inversely correlated with those of predicted target genes encoding proteins which take part in pathways controlled by toll-like receptors and interleukin-1β (Table 1). In another study, Zanette et al. analyzed patient’s primary leukemia blasts, and demonstrated that those carrying the t(8;21), which generates the most common acute myeloid leukemia-associated fusion protein AML1/ETO, exhibited low levels of miRNA-223, a regulator of myelopoesis. They showed that miR-223 is a direct transcriptional target of AML1/ETO which causes heterochromatic silencing of this miRNA by recruiting chromatin remodeling enzymes at an AML1-binding site on the pre-miR-223 gene. Ectopic miR-223 expression, RNA interference against AML1/ETO, or demethylation enhance miR-223 levels and restore cell differentiation. These findings confirm a central role of miR-223 in...
miRNAs and lymphomas

MiRNAs are involved in the pathogenesis of both Hodgkin and non-Hodgkin lymphomas. There are many studies that describe the role of miRNAs in these two groups of lymphomas.

Hodgkin lymphoma

Nie et al. showed that mir-9 and let-7a target PRDM1/Blimp-1 in Hodgkin lymphomas cell lines. In fact, the levels of mir-9 and let-7a inversely correlated with PRDM1/Blimp-1 expression in Hodgkin Lymphoma (HL) cells. Similar to their in vitro counterparts, the majority of cells in primary HL cases showed weak or no PRDM1/Blimp-1 expression. Moreover, mir-34b and CREB was also supported by full blown B-cell malignancy, providing in vivo evidence that this miRNA involve-ment in the pathogenesis of hematologic malignancies.

Navarro et al., analyzed the expression of miRNAs in 49 HL patients and 10 reactive lymph nodes. They identified 3 miRNAs discriminating HL from reactive lymph nodes and 36 miRNAs differentially expressed the nodular sclerosis and mixed cellularity subtype. The obtained results, validated in a set of different cell lines, showed that mir-96, mir-128a, and mir-128b were selectively down-regulated in HL with EBV. Moreover, only one of the miRNAs differentially expressed in EBV+ cases was encoded in the 25 miRNA that distinguish HL from reactive lymph nodes, leading to the intriguing conclusion that EBV might not be a relevant event in HL pathogenesis. Gibcus et al. described a specific miRNA expression profile in HL cell lines. By comparing HL with a panel of B-cell non-Hodgkin lymphomas, they identified a signature of HL-specific miRNAs, which included mir-17-92 cluster members, mir-16, mir-21, mir-24, and mir-155. A significant down-regulation in HL was observed for mir-150 whereas an important up-regulation in HL was shown for mir-155. Moreover, the Authors identified AGTR1, FGFR7, ZNF537, ZIC3, and IKBKE as true mir-155 target genes in HL. Interestingly, high levels of miR-155 have also been observed in the germinatal center during normal lymphopoiesis. Since HL has its origin in the lymph-nodal germinal center, the hypothesis that overexpression of miR-155 is a result of an aberrant lymphocytic block of differentiation in the germinal center is compelling.

Non-Hodgkin lymphomas

Up-regulation of miR-155 has also been described in Non Hodgkin lymphomas (NHLs), as well as in several other solid and hematologi-cal malignancies. The first hint of miR-155 involvement in lymphomagenesis derived from the observation that the final part of the B-cell integration cluster (BIC) non-coding RNA (ncRNA), where miR-155 is positioned, increases MYC-mediated lymphomagenesis in a chicken experimental model. Kluiver et al. showed that the expression of both miR-155 and its host gene is induced by protein kinase C and NF-kB in several cell lines, except Ramos, a Burkitt's lymphoma cell line, in which, for yet unclear reasons, the overexpression of BIC is not accompanied by up-regulation of miR-155. Transgenic and knockout (KO) miR-155 mice models have significantly contributed to clarify the function of this ncRNA. In Costinean's transgenic mouse model, miR-155 overexpression caused a polyclonal pre-leukemic pre-B-cell proliferation followed by full blown B-cell malignancy, providing in vivo evidence of this miRNA involve-ment in the pathogenesis of hematologic malignancies. Conversely, KO mice models showed impairment of cytokine production toward T2 lymphocyte differentiation, and compromised function of dendritic cells, confirming the data of Tili et al., who showed a central role of miR-155 in regulating the immune response.

In a subgroup of NHLs, the diffuse large B-cell lymphomas (DLBCL), miR155 up-regulation occurs in the activated B-cell phenotype (ABC-DLBCL) with respect to the germinal center B-cell-like phenotype (GCB-DLBCL). Since ABC-DLBCL and GCB-DLBCL have 5-year survival rates of 30% and 59%, respectively, miR-155 expression harbors prognostic implications. In ABC-DLBCL also high levels of miR-21 and miR-221 were described, suggesting a 3 miRNA-signature specific of the histo-type of DLBCL with severe prognosis. Roehle et al. compared the miRNAome of DLBCL, follicular lymphomas and reactive lymph nodes. In particular, they found that 4 miRNAs (namely mir-330, -17-5p, -106a and -210) can discrimi-nate DLBCL, follicular lymphomas and reactive lymph nodes with an accuracy of 98%. It is compelling that among the de-regulated miRNAs of Roehle’s work, there are miR17-5p and miR106a, members of two paralogous clusters with a documented role as oncogenes in several human malignancies.

Located at 13q31-32, miR-17-92 cluster is frequently amplified in malignant B-cell lymphomas, and is over-expressed in 65% of B-cell lymphoma patients. Overexpression of miR-17-92 in murine multipotent progenitor cells (MPPs) of MYC-transgenic mice caused an increased lymphomagenesis. Xiao et al. generated mice with high miR-17-92 lymphocytic expression and described a higher rate of lymphoproliferative disorders, autoimmune and premature death. These pathological characteristics were induced by down-regulation of the oncosuppressor genes PTEN and BIM. O’Donnell et al. showed that c-MYC activates 2 members of the cluster (namely miR-17-5p and miR-20a), which directly target EZF1, a c-MYC transactivated transcription factor promoting cell-cycle progression. In summary, by directly transactivating EZF1 and indirectly (through miR-17-92 cluster) repressing its translation, c-MYC functions as a key-regulator of cell-cycle progression. Recently, Li et al. investigated genome-wide miRNA expression and copy number in 86 DLBCLs (59 primary tumors and 27 cell lines), and identified a collection of miRNAs that robustly segregated DLBCLs into three subgroups not related to the cell-of-origin classification, extent of T-cell infiltrate and tumor site. In particular, the three newly identified subsets of DLBCLs had different prognosis and showed a markedly different MYC transcriptional activity, which was correlated with the dominance of MYC-regulated miRNAs in their expression signatures.

Finally, also miR-143 and miR-145 expression is reduced in B-cell malignancies. In Burkitt’s lymphoma Raji cell line, restoration of these two miRNAs caused a dose-dependent growth inhibitory effect linked to a down-regulation of Erk5, a MAP kinase directly targeted by miR-143 and miR145.

Conclusions

There is increasing evidence in literature to support a central role for miRNAs in the pathogenesis of hematologic malignancies. Aberrations in miRNA expression lead to abnormal gene regulation and, ultimately, to a severe alteration in proteic effectors with oncogenic and/or oncosuppressor function. From the initial contribution of high throughput techniques, which allowed the identification of the de-regulated miRNAs, currently many efforts are being made to understand the
functional implications of miRNA aberrant expression. For several hematologic malignancies, a clear diagnostic and prognostic role of miRNA de-regulation has been demonstrated. The identification of miRNA targeted mRNAs is clarifying which oncogenic pathways are affected by the leukemic/lymphomatous miRNome. This knowledge represents the essential background to the development of new miRNA-based therapeutic approaches, which combine the advantage of a physiologic molecule (such as an miRNA-derived drug), to the possibility of targeting with a single molecule several members of the same and/or of several aberrantly activated pathways in the malignancy. More studies are warranted to define how big is the impact of these tiny RNA molecules in human cancerogenesis. However, there is no doubt that these ncRNAs play a role in hematologic malignancies. Decoding this role has diagnostic, prognostic and therapeutic implications of the utmost importance.

References

8. Cai X, Hagedorn CH, Cullen BR. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA 2004;10:1957-66.
Histone deacetylase inhibitors in multiple myeloma

Sarah Deleu, Eline Menu, Eilis Van Velden, Ben Van Camp, Joanna Fraiczek, Isabelle Vande Broek, Vera Rogiers, Karin Vanderkerken

Myeloma Research Center-Brussels, Department Hematology and Immunology, Vrije Universiteit Brussel (VUB), Brussels

Department of Toxicology, Vrije Universiteit Brussel - VUB, Brussels, Belgium

Abstract

Novel drugs such as bortezomib and high-dose chemotherapy combined with stem cell transplantation improved the outcome of multiple myeloma patients in the past decade. However, multiple myeloma often remains incurable due to the development of drug resistance governed by the bone marrow microenvironment. Therefore targeting new pathways to overcome this resistance is needed. Histone deacetylase (HDAC) inhibitors represent a new class of anti-myeloma agents. Inhibiting HDACs results in histone hyperacetylation and alterations in chromatin structure, which, in turn, cause growth arrest differentiation and/or apoptosis in several tumor cells. Here we summarize the molecular actions of HDACi as a single agent or in combination with other drugs in different in vitro and in vivo myeloma models and in (pre-)clinical trials.

Introduction

Multiple myeloma (MM) is a plasma cell malignancy, characterized by an accumulation of monoclonal plasma cells in the bone marrow (BM) and high levels of monoclonal immunoglobulins or paraprotein in blood and/or urine. Complex interactions between MM cells and the BM microenvironment are required for the growth and progression of MM and result in the development of drug resistance, angiogenesis and induction of bone disease. Enhanced understanding of the interactions between MM and the BM microenvironment has led to the identification of new molecular targets. Novel therapeutic approaches target growth factors [e.g. insulin-like growth factor-I (IGF-1), interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF)], adherence molecules and signaling cascades in the MM cells such as the mitogen-activated protein kinase kinase (MEK)/extracellular signal regulated kinase (ERK)-pathway, the phosphatidylinositol-3 kinase (PI3K)/protein kinase B (Akt)-pathway, the nuclear factor κ B (NFκB)-pathway and the Wnt-pathway. Moreover, cells interacting with the MM cells in the BM, such as stromal cells, endothelial cells, osteoblasts, osteoclasts and mesenchymal stem cells are also potential targets to overcome the drug resistance against conventional chemotherapy.

MM represents 1% of all cancers and it is the second most commonly diagnosed hematologic malignancy. The incidence is higher with increasing age and is 4-5 per 100,000 individuals each year worldwide. The median age at diagnosis is 67 years. The most common clinical characteristics in MM are bone pain, anemia, recurrent infections and renal failure.

The standard induction therapy for elderly patients with symptomatic myeloma, and who are not candidates for stem cell transplantation, used to be melphalan (M) and prednisone (P). Recently, improved effects on survival have been seen in patients receiving MP combined with lenalidomide (Revlimid®) (MPR), bortezomib (Velcade®) (MPV) or thalidomide and dexamethasone. New agents such as bortezomib and lenalidomide have shown promising anti-cancer activity in pre-clinical settings and the latest clinical trials with HDACi ongoing in MM patients.

Modifications of these nucleosomes on the histone level, as well as the DNA level, can alter the chromatin state which can be open or closed. The post-translational modifications on the core histones are most common on the amino-terminal lysine rich tail which passes through and around the enveloping DNA double helix. These modifications, such as acetylation, methylation, ubiquitinylation, sumoylation, phosphorylation and glycosylation are crucial in modulating gene expression, as they affect the accessibility and interaction of DNA with other non-histone protein complexes that could contain transcriptionally co-activating or co-repressing elements. Moreover, methylation of DNA, maintained by the epigenetic enzymes, methyltransferases and demethylases, also affects the chromatin structure indirectly by recruiting protein complexes containing enzymes such as histone deacetylases (HDAC). HDAC and the opposite enzyme histone acetyltransferases (HAT) are the most studied enzymes involved in the post-translational modifications of histones. Both enzymes maintain the acetylation status of histones and non-histone proteins. HAT acetylates histones resulting in neutralizing the positive charge of histones and a more relaxed, transcriptionally active chromatin, while HDAC remove the acetyl group resulting in a more compact, transcriptionally inactive chromatin structure. Inhibiting HDAC leads to hyperacetylation of histones and results in gene expression alteration. In tumor cells, several HDAC inhibitors (HDACi) have shown promising anti-cancer activities with anti-proliferative, pro-apoptotic and anti-angiogenic properties.

This review provides an overview of the anti-myeloma activity of different HDACi in pre-clinical settings and the latest clinical trials with HDACi ongoing in MM patients.

Correspondence: Prof. Dr. K. Vanderkerken, Department of Hematology and Immunology, Vrije Universiteit Brussel (VUB), Laarbeeklaan 103, B-1090 Brussels, Belgium.

E-mail: karin.vanderkerken@vub.ac.be

Received for publication: 9 February 2009.
Revision received: 20 April 2009.
Accepted for publication: 27 April 2009.

Acknowledgment: we acknowledge grants of VUB (OZR-GOA), FWO-Vlaanderen and Stichting tegen Kanker.

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Hematology Reviews 2009; 1:e9
doi:10.4081/hrv.2009.e9
The histone deacetylase family

Eighteen HDACs have been identified in humans and are subdivided into four classes based on their homology to yeast HDACs and their enzymatic activities.29,30 Class I HDACs (1, 2, 3 and 8) are homologs to the yeast Rpd3 and can generally be detected in the nucleus. They are ubiquitously expressed in several human cell lines and tissues. Based on phylogenetic analysis, Gregoretti et al. divided class I into la (HDAC1 and 2), Ib (HDAC3) and lc (HDAC8).31 Class II HDACs (4, 5, 6, 7, 9 and 10) are related to yeast Hdal (histone deacetylase 1) and can shuttle between the nucleus and cytoplasm. This class is divided into class IIa (HDACs 4, 5, 7 and 9) and class Ib (HDAC6 and 10) which contain two deacetylase domains.32 Since HDAC6 contains a unique alpha-tubulin deacetylase (TCAD) domain, it can specifically deacetylate alpha-tubulin.33

The third class HDACs are the sirtuins (SIRT 1, 2, 3, 4, 5, 6 and 7) which are homologous to the yeast Sir2 (silent information regulator 2) family. These enzymes require nicotine adenine dinucleotide (NAD)+ for their deacetylase activity in contrast to the zinc-catalyzed mechanism used in class I and II HDACs.34 The sirtuins appear to deacetylate non-histone proteins and transcription factors including p53. They can not be inhibited by HDACi such as suberoylanilide hydroxamic acid (SAHA) or Trichostatin A (TSA).35 HDAC11 represents class IV and contains residues in the catalytic core regions similar to both class I and II enzymes but does not have strong enough identity to be placed in either class.36

HDAC inhibitors

Structural classification of HDAC inhibitors
Butyrate and TSA were among the first chemicals to be identified as HDAC inhibitors. Dimethylsulfoxide was used to aid superinfection of murine erythroleukemia cells with the Friend virus, whereas TSA was originally isolated as an antifungal agent from culture medium of Streptomyces hygroscopicus. Later on, it was discovered that these compounds could induce cell differentiation and a correlation with histone hyperacetylation, which was maintained by inhibiting HDACs, could be shown.35,36 It subsequently opened a new field of research. Since then, a large number of natural and synthetic HDACi have been developed by several companies and used as anti-tumor agents in pre-clinical and clinical settings (Table 1). On the basis of their chemical structure, major HDACi can be divided into four categories: short-chain fatty acids, hydroxamates, benzamides and cyclic tetrapeptides.23,40,47

Among the various classes of HDACi, short chain fatty acids such as phenylbutyrate, the anti-epileptic drug valproic acid (VPA) and sodium butyrate are only effective at mM concentrations and thereby form the less potent class of HDACi.48 Clinical evaluations have been performed with these compounds either alone or in combination and are well tolerated in patients. However due to the short plasma half-life, high doses are needed to obtain a therapeutic effect.49 The first natural hydroxamate was TSA and is now considered as the reference compound of hydroxamate based inhibitors. Most of the synthetic hydroxamate based HDACi target class I and class II with high potency. SAHA has a potency at µM range and has recently been approved for the treatment of cutaneous T-cell lymphoma. M-carboxyaminic acid bishydroxamide (CBHA) is another potent second generation inhibitor which is the structural basis for example LAQ824 and PXD101, both effective at nM range towards classes I and II. Two of the newest hydroxamates based HDACi are LBH589 and ITF2357 with very low IC50 values at nM concentrations.46 Benazamides include MS-275 and CI-994 and are generally less potent than the hydroxamates and cyclic tetrapeptides. Cyclic tetrapeptides, include the natural product depsipeptide (FK 228 or FR 901228) and apicidin. Depsipeptide is a prodrug and has to be metabolically activated via reduction and dephosphorylation.47 Recently KD5170, a novel mercaptotetone-based histone deacetylase inhibitor, has been developed. KD5170 showed significant anti-proliferative activity against a variety of human tumor cell lines, including human MM cell lines.48

Isoenzyme-selectivity of pan-HDACi and mechanism of HDAC inhibition
In general, none of these inhibitors, except tubacin, exhibit specificity towards one isoform. However, they inhibit the enzyme activity of HDACs with varying efficiency (Table 1). For example, depsipeptide preferentially inhibits HDAC1 and 2 compared to HDAC4 and 6, whereas the potency of MS-275 to inhibit HDAC6 is 26 times higher compared to HDAC3 and appears to lack the ability to inhibit the HDAC6 and 8.46 Tubacin, the HDAC5 selective inhibitor, induces hyperacetylation of tubulin and has no effect on the histone acetylation status, whereas other hydroxamate inhibitors such as TSA, SAHA and LBH589 induce histone – and tubulin hyperacetylation.48-50

X-ray crystallographic analyses clarify the mechanism of HDAC enzyme using an HDAC-like protein (HDLP) isolated from an anaerobic bacterium, on the one hand and on the other hand how inhibitors such as SAHA and TSA mediate HDAC inhibition. The HDAC catalytic domain consists of a tube like pocket whereby a Zn2+ cation is positioned near the bottom of this narrow pocket. The basic structure of the HDACi contains a cap group, an aliphatic chain for a spacer, and a functional group (except depsipeptide). The cap group may be necessary for packing the inhibitor at the rim of the tube-like active site pocket, while the aliphatic group forms interactions with the residues of the lining pocket. For TSA, the hydroxamic acid group (the functional group) coordinates the zinc through its carboxyl and hydroxyl groups, resulting in the formation of a penta-coordinated zinc and thereby altering the activity of the enzyme.51,52

Pre-clinical observations of HDACi in multiple myeloma

Anti-myeloma activity of HDACi as a single agent in vitro
HDACi modulate the gene expression profile of multiple myeloma cells
Microarray based studies showed that HDACi induce transcriptional modulations of 7-10 % of the genes in malignant cell lines by acetylation of histones and non-histone proteins.53-55 The patterns of the HDACi induced gene expression alterations are quite similar for different HDAC inhibitors. Definite differences, however, could be observed by different agents in different cancer cell lines.53-55

In MM, the first cDNA array using SAHA in the human MMIS cell line was performed by Mitsiades et al. SAHA caused selective gene expression alterations of oncogenes, proliferative/anti-apoptotic transcription factors, cell cycle regulators and members of the IGF-IR and IL-6R signaling cascades.56 Recently, gene expression profiling of MMIS cells exposed to VPA have also been performed and showed that VPA also targeted genes involved in the cellular pathways crucial for the survival of the MM cells as seen for SAHA. Furthermore, they could demonstrate modulation of genes that contribute to RNA splicing/transcription and DNA replication, indicating that HDACi could affect cell growth differently from apoptosis or cell cycle regulation.56

HDACi inhibit the proliferation of multiple myeloma cells

Before investigating the molecular effect of HDACi in certain human MM cell lines, assays such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)- or 3H-thymidine incorporation assays were performed to study the anti-proliferative effect of the HDACi. Table 2 shows an overview of different HDACi and their concentration range needed to inhibit the proliferation of the human MM cell lines and/or primary human MM cells. HDACi such as VPA, FK228 and ITF2357 affected the viability of IL-6 dependent as well as IL-6 independent MM cell lines, indicating
Table 2. Potency of HDACi used in different in vitro MM models.

<table>
<thead>
<tr>
<th>HDACi</th>
<th>Range</th>
<th>MM cells</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaN</td>
<td>mM</td>
<td>U266, RPMI 8266, ARH-77, OPM2</td>
<td>60</td>
</tr>
<tr>
<td>VPA</td>
<td>mM</td>
<td>OPM1, MM15, DOX-40, INA-6, OPM2, NCI-H929, LP-1, RPMI 8266, U266</td>
<td>56, 61, 62, 63</td>
</tr>
<tr>
<td>SAHA</td>
<td>pM</td>
<td>MM1S</td>
<td>55, 64</td>
</tr>
<tr>
<td>LAQ824</td>
<td>Sub-pM</td>
<td>primary human MM cells, MM1S, MM1R, RPMI 8266, ARH-77, OPM2</td>
<td>65</td>
</tr>
<tr>
<td>KD5170</td>
<td>R</td>
<td>H299, U266, primary human MM cells</td>
<td>66</td>
</tr>
<tr>
<td>FK228</td>
<td>nM</td>
<td>U266, RPMI 8266</td>
<td>67</td>
</tr>
<tr>
<td>ITF2357</td>
<td>nM</td>
<td>CMA-03</td>
<td>68</td>
</tr>
<tr>
<td>LBH589</td>
<td>nM</td>
<td>primary human MM cells, MM1S, MM1R, U266, LRT-40, DOX-40</td>
<td>69</td>
</tr>
</tbody>
</table>

MM1S: dexamethasone-resistant; MM1R: dexamethasone-resistant; DOX-40: doxorubicin-resistant; IL-6-independent; IL-6: interleukin-6; OA: DMSO; CMA-03: doxorubicin-resistant; MM1S: IL-6 independent; OPM2: IL-6-dependent; dexamethasone-resistant when IL-6 is added; ARH-77: Epstein-Barr virus (EBV) positive cell line and thereby not considered as a genuine MM cell line; S: sensitive; R: resistant.

Table 2. Isoenzyme-selectivity of pan-HDACi.

<table>
<thead>
<tr>
<th>Class</th>
<th>Compound</th>
<th>HDAC specificity</th>
<th>Company/Sponsor</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short-chain fatty acid</td>
<td>Butyrate</td>
<td>Class I, IIa</td>
<td>Merck</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Valproic acid</td>
<td>Class I, IIa</td>
<td>NCI</td>
<td>41</td>
</tr>
<tr>
<td>Hydroxamate</td>
<td>SAHA</td>
<td>Class I, II</td>
<td>Merck</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>PXD010</td>
<td>Class I, II</td>
<td>Curagen</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>LAQ824</td>
<td>Class I, II</td>
<td>Novartis</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>LBH589</td>
<td>Class I, II</td>
<td>Novartis</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Tubacin</td>
<td>Class IIb</td>
<td>BI and MIT</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>ITF2357</td>
<td>Class I, II</td>
<td>Italfarmaco</td>
<td>43</td>
</tr>
<tr>
<td>Mercaptopetone</td>
<td>KD5170</td>
<td>Class I, II</td>
<td>Kalypsys</td>
<td>44</td>
</tr>
<tr>
<td>Cyclic tetrapeptide</td>
<td>Depsipeptide</td>
<td>Class I</td>
<td>Gloucester</td>
<td>45</td>
</tr>
</tbody>
</table>

NCI: National Cancer Institute; BI: Biomedical Institute; MIT Massachusetts Institute of Technology.

that the anti-myeloma activity of the HDACi is not influenced by one of the key growth factors in MM.60,61,62 Furthermore, co-culturing the MM cells with bone marrow stromal cells (BMSC) does not protect the cells from cell death induced by the HDACi LAQ824, ITF2357, LBH589 or KD5170.63,64,65,66,67 These data suggest that HDACi could overcome the protective effect of the BM micro-environment. The MM1S cells were resistant to KD5170 and showed no increase in histone acetylation, whereas KD5170 sensitive cell lines exhibited histone hyperacetylation after KD5170 treatment.67 This finding indicates that inhibition of the HDAC enzymes is necessary for the anti-tumor effects of the HDACi.

JNJ-26481585, a recently developed novel hydroxamate based HDACi with prolonged pharmacodynamic properties, has anti-proliferative effect at nM concentrations in the murine ST33M model.68 This murine MM cell line is derived from the STMM mouse model which mimics the human disease closely at the molecular, cellular and clinical level.69,70 HDACi induce cell death in multiple myeloma

Besides inhibition of proliferation, HDACi induced cell death is one of the major mechanisms to inhibit the survival of the myeloma cells. Extrinsic and intrinsic apoptotic pathways as well as non-apoptotic cell death such as autophagy have been reported in myeloma cells treated with an HDACi. Figure 1 demonstrates effects of the HDACi on the compounds of the intrinsic and extrinsic apoptotic pathway. The extrinsic apoptotic pathway is activated by ligand binding to death receptors such as Fas (APO-1 or CD95), tumor necrosis factor receptor-1 (TNFR-1) and TNF-related apoptosis inducing ligand (TRAIL or Apo2L) receptors (DR4 and -5), resulting in activation of caspase-8 and caspase-10. Apo2L/TRAIL interacts with two death receptors (DR4 and DR5) and potently induces apoptosis in various tumors, including primary MM cells and MM cell lines, while exerting minimal or no toxicity in normal cells.71,72 Several studies have demonstrated that HDACi can upregulate the expression of both death receptors and their ligands and are proposed to occur selectively in tumor cells.73 The U266 human MM cell line, although expressing significant levels of DR4 and caspase-8, is resistant to Apo2L/TRAIL and this resistance could be overcome with VPA. This sensitizing effect of VPA is mediated by the redistribution of DR4 to lipid rafts followed by an improved DR4 signaling.74 However, opposite results have been obtained by Schwartz et al. who have demonstrated that VPA activated caspase-3 but not caspase-9 and caspase-8 in the U266, OPM2 and RPMI human MM cell lines.75 In the MM1S line, treated with LBH589, no upregulation of death receptors and their ligands could be observed. Caspase-8, however, was activated and the gene expression of the TOGA gene, negative regulator of the Fas ligand (FasL) or TRAIL induced apoptosis was downregulated.76 SAHA sensitized MM1S cells to a Fas-activating monoclonal antibody CH-11 and to recombinant TRAIL. This sensitizing effect was associated with decreased expression of the anti-apoptotic protein FLICE-like inhibitory protein (FLIP) and members of the inhibitors of apoptosis (IAP) family such as X-linked IAP (XIAP).77 Despite these results showing that HDACi affect the extrinsic pathway, in MM and other malignant cells it is still not clear how important the death-receptor pathway is for the therapeutic effects of HDACi.

The intrinsic apoptotic pathway is mediated by the mitochondria whereby proapoptotic signals result in the release of mitochondrial intermembrane proteins, such as cytochrome c (cyto-c), apoptosis inducing factors (AIF) and second mitochondria-derived activator of caspase (Smac). Cytosolic cyto-c binds to apoptotic protease activating factor (Apaf-1), resulting in Apaf-1 oligomerization and subsequent caspase-9 activation while cytosolic Smac binds to XIAP and thereby eliminates its inhibitory effect on caspase-9. Cytosolic AIF induces caspase-independent apoptosis.78 Members from the BCL2 family partially regulating this pathway, contain the pro-apoptotic (e.g. Bax, Bak, Bid and Bim) and anti-apoptotic (e.g. Bcl2, BclXL and Mcl1) proteins. The BCL2 protein Bid, can be cleaved by caspase-8 after death-receptor ligation, and truncated Bid (tBid) localizes to the mitochondria to initiate the intrinsic apoptotic pathway.79 MM cells contain higher levels of the anti-apoptotic proteins Bcl2 and Mcl1 and lower levels of the pro-apoptotic protein Bax compared to normal plasma cells.80,81 These findings could play a role in the survival of the MM cells and the resistance to chemotherapeutic agents.

How HDACi activate the intrinsic apoptotic cascade is cell context dependent and is still not completely understood. Treatment of the U266 human MM cell line and primary MM human cells with depsipeptide resulted in a decrease of the anti-apoptotic proteins Mcl1,
cell death is calpain-independent and partially
strate in MM1S cells that the LBH589 induced
methyle ketone (z-VAD-fmk), they could demon-

Novel apoptosis and caspase activation

Figure 1. Induction of the extrinsic and intrinsic apoptotic pathway by HDACi in myelo-

ma cells. The extrinsic apoptotic pathway is triggered by ligand binding and leads to activ-

ation of caspase-8, which, in turn, activates caspase-3. Activation of the intrinsic apopto-

tic pathway results in the release of three compounds: (a) cytochrome-c (cyto-c) which

binds to apoptotic protease activating factor (Apaf-1) to activate caspase-9, (b) apoptosis

inducing factors (AIF) and (c) second mitochondria-derived activator of caspase (Smac).

FLICE-like inhibitory protein (FLIP) and members to the inhibitors of apoptosis (IAP)

are able to prevent apoptosis induced by death receptors or intrinsic pathway respective-

ly. Symbols denote compounds that are up-regulated (*), down-regulated (**), activated

(>) or translocated to cytosol (\(\triangle\)) by HDACi in myeloma cells.

Bcl2, BclxL and an increase in Bax; this latter could only be observed in primary human MM
cells.65 ITF2357 induced apoptosis through the intrinsic pathway rather than through the
extrinsic pathway in the KMS18 MM cell line since no cleavage of caspase-8 nor upregula-
tion of DR-4 have been found, whereas cleavage of caspase-3 and -9 and downregulation of
Bcl2 and Mcl1 could be demonstrated.66 MM1S cells treated with LBH589 underwent translo-
cation of cyto-c and AIF from the mitochondria to the cytosol, upregulation of Apaf-1 and cleav-
age of Bid, caspase-9 and caspase-3. Furthermore, gene expression profiling revealed a

novel apoptosis and caspase activation inhibitor, AVEN, which was downregulated by
treatment with LBH589.67 These data represent clear evidence that LBH589 caused cell death
through mitochondrial perturbations. Both LBH589 and SAHA induced poly (ADP-ribose)
polymerase (PARP) cleavage in MM cells by two different enzymes, caspase-3 and calpain,
respectively. Using the calpain inhibitor, calpeptin, and the caspase-3 inhibitor, benzyl-
loxy carbonyl-Val-Ala-Asp methylester-fluoro methylketone (z-VAD-fmk), they could demon-
strate in MM1S cells that the LBH589 induced cell death is calpain-independent and partially
caspase-dependent, while the SAHA induced cell death is calpain-dependent and caspase-
dependent.68,69 Furthermore, SAHA promotes cleavage of Bid to BiBid while overexpression of
the anti-apoptotic protein BcI2 inhibited SAHA-induced apoptotic signaling.68 Recent
data indicate that KD5170 mediates cell death through mitochondrial perturbation in the
U266 cells. KD5170 provoked Bax activation and cleavage of caspase-9 and caspase-3, caus-
ing loss of mitochondrial membrane potential and subsequent pro-apoptotic factor release.
The fact that AIF was released, and that the nuclear condensation was partially blocked in
cells pre-treated with z-VAD-fmk before exposure to the HDACi, suggest that KD5170
induced apoptosis through both caspase-dependent and caspase-independent pathways.
Furthermore, KD5170 induced oxidative stress and oxidative DNA damage in myeloma
cells as evidenced by the upregulation of heme oxygenase-1 and H2AX phosphorylation,
which is a marker of DNA double strand breaks.68,69

Autophagy, an alternative model for apopto-
sis, has been reported to contribute to the
HDACi induced cell death in several tumor cell
lines.62,63 Autophagy is a catabolic process
involving the degradation of long-lived pro-
teins or cytoplasmic organelles through the
lysosomal machinery.64 Schwartz et al. demon-
strated for the first time that autophagy might
be involved in VPA induced cytoproticity in
human myeloma cell lines. Only cleavage of
caspase-3 and autophagic vacuoles in the cyto-
plasm could be observed in the myeloma cells
treated with VPA, indicating that autophagic
cell death might be involved.65

HDACi induce cell cycle arrest

HDACi, except tubacin, induce cell cycle
arrest at G1/S phase. The events in the G1
phase are coordinated by the three early G1
cyclins (1, 2, 3) and their associated cyclin-
dependent kinases (CDKs) 4/6 (G1 progression)
and CDK 2 (G1/S transition). The transcrip-
tional regulation of the genes, necessary
for G1 progression and G1/S transition,
depends on the phosphorylation state of the
retinoblastoma (Rb) protein. Phosphorylation
of the Rb protein by G1 cyclin/ cyclin-depend-
ent kinase (CDK) results in the release of E2F,
allowing transcription activation and further
progression through G1 and initiation of S
phase. The CDK inhibitors, including the INK4
family (p16) and the Cip/Kip family (p21, p27
and p57), are proteins that negatively regulate
the cell cycle by competing with the cyclin D
-CDK binding and therefore inhibiting the CDK
complex kinase activity. In MM, constitutive
phosphorylation of the Rb protein may be fund-
amental to the growth and development of the
tumor.70 The mRNA level of the three G1 D
cyclins are elevated in virtually all MM tumors
compared to healthy plasma cells and could be
due to an Ig translocation or an unknown
mechanism. The elevated levels of the D
cyclins are not sufficient to promote a cell cycle
and need a corresponding increase of CDK4 or
CDK6.64 Furthermore, several reports have
demonstrated that p16 is frequently hyperme-
thylated in primary human MM cells. However,
no decreased mRNA could be found.65,67

HDACi induce cell cycle arrest in the G1/S
phase which is mostly associated with induc-
tion of p21. This has been observed in the MM
cell lines treated with VPA, NVP-LAQ824,
LBH589, NaB, SAHA and ITF2357.6,14,45,66,67,69 MM
cells treated with VPA or LBH589 also showed
a reduction of cyclin D1 and/or cyclin D2, indi-
cating that induction of p21 is not solely
responsible for cycle arrest.66,67

HDACi inhibit the aggresomal pathway
in multiple myeloma

The aggresomal protein degradation system
represents an alternative system to the protea-
some for degradation of polyubiquitinated mis-
folded/unfolded proteins (Figure 2).7 When
degradation of misfolded proteins exceeds the
proteasomal degradation through e.g. protea-
some inhibitors, proteins interact with other
unfolded or partially folded proteins, resulting

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Hematology Reviews 2009; 1:e9
in accumulation of ubiquitylated proteins, organized into perinuclear structures termed “aggresomes”. Aggresomes are formed by the retrograde transport of the aggregated proteins on microtubules (MT) and travel to the MT organizing center (MTOC) region, where they are sequestered as a single structure susceptible for lysosomal degradation. Movement of aggresomes requires intact microtubules and association with motor dynein.

HDAC6 deacetylates alfa-tubulin and plays a key role in the aggresomal pathway since it can bind polyubiquitinated proteins and dynein, facilitating the transport of aggresomes along the MTs. Targeting HDAC6 with tubacin or pan HDAC inhibitors such as SAHA or LBH589, results in hyperacetylation of alfa-tubulin, accumulation of polyubiquitinated proteins and apoptosis. It has been shown that tubacin inhibits MM cell growth in drug-sensitive (MM1S, U266, INA-6 and RPMI8226) and drug-resistant cell lines (RPMI-LR5 and RPMI-Dox40) with an IC_{50} between 5-20 µM, whereas no cytotoxicity in peripheral blood mononuclear cells (PBMCs) could be observed at µM levels. This indicates that tubacin sensitivity is independent of drug resistance and that tubacin selectively targets malignant cells.

**HDACi affect cytokines and proteins implicated in multiple myeloma survival, progression and immune escape**

Mitsiades et al. showed that SAHA suppresses the expression of receptor genes involved in MM cell proliferation, survival and/or migration such as IGF-1R, IL-6R and its key signal transducer gp130, TNF-R, CD138 (syndecan-1) and CXCR-4. Furthermore, in MM1S cells they could demonstrate that SAHA suppressed autocrine IGF-1 production and paracrine IL-6 secretion of BMSC by triggering MM cell binding. This suggests that SAHA can overcome cell adhesion-mediated drug resistance. OPM-2 cells treated with NaB decreased IL-6R but when cells were transfected with an expression vector of IL-6R no decrease of the receptor could be observed. Increased p21 expression and apoptosis could be observed in both transfected and untransfected cell lines, indicating that downregulation of the IL-6R is not required for the induction of p21 or apoptosis. This observation again confirms that HDACi act on multiple cellular pathways.

Several studies provide evidence that HDACi suppress angiogenesis through a direct effect on the growth and differentiation of endothelial cells on one hand and by down-regulating the expression of pro-angiogenic genes in tumor cells on the other hand. The anti-angiogenic effect of HDACi in myeloma has been demonstrated using OPM-2 and KMS cells treated with VPA. VPA decreases VEGF secretion and VEGF receptor expression, resulting in inhibition of the vascular tubule formation of endothelial cells in co-cultures with myeloma cells. These data confirm the anti-angiogenic effect of HDACi on myeloma which is important to suppress spread of the MM cells.

Recently, De Bruyne et al. showed that the tetraspanin CD9 which shows an inverse correlation between its expression level and tumor metastasis in solid tumors, is epigenetically down-regulated in MM and could be up-regulated by treating myeloma cells with LBH589. Myeloma cells expressing CD9 become more susceptible for natural killer mediated cytolysis and the expression correlates with non-active MM disease. These observations suggest that the immune escape of the tumor cells and molecules, correlating with the MM disease status, can be affected by HDACi.

**Anti-myeloma activity of HDACi in combination therapy in vitro**

**Bortezomib**

Bortezomib, a first-in-class, potent and reversible proteasome inhibitor, has been successfully introduced in clinical practice and represents the standard of care in symptomatic MM patients. The anti-myeloma activity of bortezomib is a result of NF-κB inhibition, upregulation of various apoptotic pathways, and effects on the tumor micro-environment. Pei et al. were the first to demonstrate in vitro that HDACi in combination with bortezomib resulted in an improved cytotytic effect compared to their effect as single agent. Sequential exposure of U266 and MM1S cells to bortezomib and SAHA or NaB potently induced caspase-3, -8 and -9 activation and release of the pro-apoptotic mitochondrial proteins cyto-c and Smac, resulting in a synergistic induction of apoptosis. This effect was associated with a reduction in NF-κB DNA binding activity, modulation of JNK activation and a reactive oxygen species (ROS)-dependent downregulation of Cyclin D1, Mcl-1 and XIAP. Combining bortezomib with PXD101 caused oxidative stress accompanied by an
enhanced effect on Bim expression, DNA damage, MAPK p38 activation and p53 phosphorylation. These observations indicate that there are several molecular mechanisms that may contribute to the synergy between bortezomib and HDACi. Specific inhibition of the aggresomal pathway by tubacin together with proteasome inhibition by bortezomib also resulted in an accumulation of ubiquitinated proteins followed by a synergistic anti-MM-activity, mediated by stress-induced JNK activation, followed by caspase/PARP cleavage. In addition, further investigations on cytoskeletal events showed that bortezomib alone leads to aggresome formation and, combining it with LBH589 or SAHA, both inhibiting HDAC6, resulted in a disruption of aggresome formation leading to apoptosis. Nawrocki et al. demonstrated that the oncogen Myc regulates the sensitivity of MM cells to bortezomib in combination with SAHA. Oncogenic activation of Myc is a hallmark of nearly all rapidly dividing malignant cells. In MM, the Myc expression is directly correlated with intracellular endoplasmatic reticulum (ER) content and protein synthesis rate. Bortezomib in combination with SAHA resulted in an induction of the pro-apoptotic BH3-only protein Noxa and ER stress indicated by a disruption of calcium homeostasis and activation of caspase-4. Further knock-down studies demonstrated that caspase-4 and Noxa play significant roles in Myc-driven sensitivity to the combination of bortezomib and SAHA.

Enhanced anti-MM activity of the combination therapy could not only be observed in primary human MM cells but also in co-culture conditions and conditions with exogenous growth factors IL-6 or IGF-1. Taken together, bortezomib in combination with HDACi may represent a promising therapeutic strategy that can overcome drug-resistance.

Death receptor ligands

In several tumor cells, an enhanced apoptotic effect can be observed using HDACi and activators of the TRAIL and Fas pathway. However, the molecular mechanism underlying this synergism is still unclear and is cell-type specific.

Fandy et al. demonstrated that TSA, as well as SAHA, in combination with TRAIL have potent synergistic effect in the ARO-1 MM cells. Similar apoptotic effects have been observed in MM1S, U266 and H929 cell lines treated with KD5170 and TRAIL. The fact that SAHA and TSA could up-regulate the two death receptors DR4 and DR5 in the MM cells, coupled with a downregulation of anti-apoptotic proteins (Bel-2 and XIAP) could explain the synergistic effect of combination therapy. However, it has been shown that HDACi could also achieve synergy with TRAIL without changing the TRAIL receptors or anti-apoptotic proteins, by simultaneously activating the intrinsic and extrinsic pathways.

DNA methyltransferase inhibitors

5-azacitidine is a DNA methyltransferase inhibitor and shows activity against MM. 5-azacitidine and analogs such as 5-azacytidine (decitabine) are interesting tools to investigate hypermethylation in tumorigenesis and the clinical efficacy is currently being assessed in phase II trials. Several investigations have already shown that hypermethylated tumor suppressor genes can be most efficiently reactivated by combining DNA demethylating agents with HDACi, this could thereby result in an enhanced reduction of tumor cell growth.

Treatment of the human myeloma cell line, U266 with NaB and decitabine resulted in a G1 arrest, whereas no cell cycle arrest could be observed when the compounds were used as single agents. Also, the expression level of the p16 gene on RNA and protein level was significantly increased when both epigenetic agents were applied simultaneously. Our group could also show in the human myeloma Karpas707 cell line that the upregulation of the pro-apoptotic protein Bim by LBH589 could be enhanced by decitabine, while decitabine alone had no effect on Bim expression.

Conventional therapeutic agents

LAQ824, depsipeptide and LBH589 showed an enhanced decrease in survival of human MM cell lines with the conventional therapeutic agents such as dexamethasone and melphalan. Targeting different pathways could contribute partially to the enhanced anti-MM effect; namely caspase-8 is activated by LAQ824 and not by dexamethasone whereby combining both agents provides an additional apoptotic signal to those already induced by dexamethasone. Further investigations are needed to clarify the molecular mechanism of the synergism between chemotherapeutic agents and HDACi.

Anti-myeloma activity of HDACi in vivo

To study the pathogenesis of MM and to find new treatment strategies, different animal models have been developed, each with their own advantages and disadvantages.

To determine whether in vivo the anti-myeloma effects of LAQ824, VPA and KD5170 correlate with their in vitro activity, human MM xenografts in immunodeficient mice were used. Xenograft murine models were subcutaneously injected with RPMI8226, OPM1 or H929 and daily treatment with LAQ824, VPA or KD5170, respectively, started when tumors were measurable. These in vivo studies resulted in a significant decrease in tumor growth and a significant increase in survival of mice treated with the HDACi.

Furthermore, the enhanced anti-myeloma activity of LBH589 with bortezomib could be demonstrated in vivo by Atadja et al. using a disseminated luciferized MM15 MM xenograft mouse model. One of the major limitations of these in vivo experiments is the lack of the interaction of MM cells with a human micro-environment and therefore a protective effect of the BM micro-environment against the anti-myeloma activity of the HDACi in vivo cannot be excluded.

Recently, the syngeneic murine 5T3 and 5T2MM models, which mimic the human myeloma disease closely, have been used to investigate the anti-myeloma activity of JNJ-26481585. Injecting C57B/KaLwRij mice with 5T2 or 5T33MM cells results in a migration of the MM cells to the BM followed by tumor growth, induction of angiogenesis and induction of a MM bone disease (only in the 5T2MM model). 5T2 and 5T33MM cells treated with JNJ-26481585 resulted in a significant decrease in tumor load and a reduction in the MM bone disease. Moreover, when a very low dose of JNJ-26481585 was combined with bortezomib, MM bone disease was more reduced than seen with bortezomib alone (Deleu et al., personal observations, 2009). These in vivo studies demonstrated that the antimyeloma activity of the HDACi as single agents or in combination with bortezomib could not be overcome by the BM micro-environment.

Clinical observations of HDACi in multiple myeloma

Several clinical trials with HDACi alone or in combination with other antiymeloma agents are ongoing (Table 3). Phase I clinical trials showed that HDACi, such as SAHA, LBH589 and depsipeptide are well tolerated in myeloma patients. In phase II clinical trials, the activity of the HDACi as single agent was limited. However, combining HDACi with dexamethasone and/or bortezomib resulted in a more promising therapeutic setting in the treatment of MM, even in patients with refractory and relapsed MM.

Future directions

It has become clear that pan-HDACi have anti-neoplastic activities by affecting multiple pathways involved in cell growth, survival, immune response and tumor vasculature. However, the precise underlying mechanism of the inhibition of the different HDACs by pan-HDACi and their biological role in MM pathogenesis remain to be clarified. A greater
understanding of the molecular effects of the HDACi and the role of HDACs is essential in selecting patients who are potential candidates for HDACi therapy and in designing combination studies. The development of isoform-specific inhibitors would be a valuable tool to investigate the biological role of specific HDACs. However, it is still not clear whether selective inhibition of HDACs has therapeutic advantages over a pan-HDACi. Clinical trials demonstrated promising anti-tumor responses to HDACi, mainly in combination with other agents such as bortezomib or dexamethasone which are already in clinical use. Therefore, the development of new and improved HDACi should be encouraged together with their use in combination therapy to improve the outcome for MM patients.

### References


37. CAHA+bortezomib Phase I 122

38. SAHA+bortezomib Phase I 123, 124

39. ITF2357 Phase II 125

### Table 3. Ongoing clinical trials with HDACi as single agent or in combination therapy in MM patients.

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Clinical trial</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depsipeptide+bortezomib+dexamethasone</td>
<td>Phase I/II</td>
<td>118</td>
</tr>
<tr>
<td>LBH589</td>
<td>Phase I/II</td>
<td>119, 120</td>
</tr>
<tr>
<td>LBH589+bortezomib</td>
<td>Phase I</td>
<td>121</td>
</tr>
<tr>
<td>SAHA</td>
<td>Phase I</td>
<td>122</td>
</tr>
<tr>
<td>SAHA+bortezomib</td>
<td>Phase I</td>
<td>123, 124</td>
</tr>
<tr>
<td>ITF2357</td>
<td>Phase II</td>
<td>125</td>
</tr>
</tbody>
</table>


The role of JAK2 abnormalities in hematologic neoplasms

Mohammed K. Alabdulaali
Blood Diseases Center, King Fahad Hofuf Hospital, Hassa, Saudi Arabia

Abstract

In 2005, an activating mutation in the Janus kinase 2 (JAK2) was identified in a significant proportion of patients with myeloproliferative neoplasms, mainly polycythemia vera, essential thrombocythemia and primary myelofibrosis. Many types of mutations in the JAK-STAT pathway have been identified, the majority are related to JAK2. Currently JAK2 mutations are important in the area of diagnosis of myeloid neoplasms, but its role beyond the confirmation of clonality is growing and widening our knowledge about these disorders. In addition to that, clinical trials to target JAK2-STAT pathway will widen our knowledge and hopefully will offer more therapeutic options. In this review, we will discuss the role of JAK2 abnormalities in the pathogenesis, diagnosis, classification, severity and management of hematologic neoplasms.

Classification of myeloid neoplasms

As JAK2 abnormalities are mainly identified in myeloid neoplasms, we will summarize the current classification scheme for these disorders. In general they are divided into 3 major groups: acute leukemia, chronic leukemia and myelodysplastic syndrome. Chronic leukemia can be sub-divided into BCR/ABL positive or negative, whereas BCR/ABL negative will be divided further into classical MPNs, non-classical MPNs with or without dysplasia, and myelodysplastic syndrome (MDS) (Figure 1). The diagnosis and classification of these disorders are based on peripheral blood counts, blast percentage, type of myelosis, presence of significant dysplasia, extent of fibrosis, clinical features, biochemistry and most importantly genetics.24 JAK2 abnormalities are not only associated with most of the classical myeloid neoplasms but they are also seen in association with other myeloid neoplasms except BCR/ABL positive CML and acute lymphoid leukemia (ALL) where it is only rarely reported as we will see later on. One of the interesting things regarding the clinical features of myeloid neoplasms is their tendency to transform to acute leukemia; the classical MPNs, beside their pre-leukemic behavior, progress and regress to each other (Figure 2). So, why does an abnormal gene give rise to different disorders? Why do classical MPNs progress and regress? What are the roles of JAK2 abnormalities in the pathogenesis of hematologic neoplasms?

JAK family

Janus kinase is a family of intracellular non-receptor tyrosine kinases that transduce cytokine-mediated signals. At present, it consists of 4 members: JAK1, JAK2, JAK3 and TYK2.6 Janus kinases were named after Janus or Ianus who in Roman mythology was believed to be the god of beginnings, god of new births and endings. He was imagined as having two faces or heads facing in opposite directions.7 Indeed, Janus kinases are located just beneath the cellular receptors to control the signal transmission downstream and have seven domains, two of which are structurally similar. One of these (JH1) is an activating domain while the other (JH2) seems to exert an inhibitory effect (Figure 3).

Upon ligand binding to its specific receptor, JAK protein will be activated and it will then phosphorylate the downstream signaling molecules like STATs, which will be actively transported to the nucleus where it will activate transcription factors (Figure 4).

Abnormalities in JAK1 have been reported in ALL of mainly T cell type where it is found in nearly 20% of the cases,6 JAK2 is found in myeloid neoplasms and rarely rearranged in ALL,4 JAK3 have been reported in more than 50% of transient abnormal myeloipoiesis in Down syndrome patients, acute myeloid leukemia (AML) megakaryoblastic type (M7) and in some cases of severe combined immune deficiency (SCID).56 TYK2 might have a role in lymphoid neoplasms and natural killer cell functional defects.7

JAK2

JAK2 was mapped on the short arm of chromosome 9p24 in 1992 by Pritchard and his colleagues.8 It has 140 kb spanning 25 exons to form 1132 aminoacid JAK2 protein.14 It works as a signaling molecule for many cytokines including: INF-γ,15 erythropoietin (EPO), prolactin,7 thrombopoietin (TMP), G-CSF, GM-CSF8 and IL-36 via activating many signaling pathways like: MAPK, P38, ERK2 and STATs.14 PRV1 (CD177) and NF-E2

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also appear to be activated and over-expressed by JAK2. One of the most important pathways that are activated by JAK2 is STAT5 followed by activation of BCL-XL and finally upregulation of BCL2 where the cell will gain a survival advantage.

Types of JAK2 abnormalities and pathogenesis

In general we can divide these into 4 categories:

A. Rearrangements

JAK2 can be rearranged to:
- TEL/ETV6: t(9;12) (p24;p13) reported in some CML like MPNs and T-cell ALL;
- BCR: t(9;22) (p24;q11.2) reported in some CML like MPNs;
- P harm1: t(8;9) (p22;p24) reported in some MPNs, AML and ALL;
- NF-E2: der(9) t(9;12) (p24;q13) reported in some cases of MDS.

B. Point mutations

- V617F G >T at nucleotide 1849 on exon14, reported in mainly the classical MPNs;
- T875N reported in AML (M7);
- R683G and less frequently other R683 point mutations reported in 18-28% of ALL in Down syndrome patients and 10% of a high-risk cohort of childhood ALL in patients without Down syndrome.

C. Deletions/Insertions

- Exon12: there are more than eight reported mutations including deletions and insertions in 538 to 543 codons reported in 4% of PV cases;
- IREED del which is a five amino acids deletion in JH2 pseudokinase domain reported in B-cell ALL in Down syndrome patients.

D. Numerical

- It can present as trisomy (+9) or be over-expressed due to amplification.

The majority of these abnormalities are affecting the JH2 domain leading to loss of inhibitory effects on JH1 domain, hence the later will be auto-activated. Suppressors of cytokine signaling (SOCS) 1 and 3 are negative regulators for JAK2 kinase, these suppressors will also be phosphorylated and stabilized by the hyperactive tyrosine kinase. SOCS3 promoter methylation is another mechanism that is found in a group of patients.

It is reported that 5-10% of MPN patients have at least one relative who is affected by this disorder, and in familial MPNs the risk of developing these disorders increases 6-fold. Recent studies suggest that particular single nucleotide polymorphisms (SNP) are associated with a higher risk of developing JAK2 V617F mutation. Out of about 659 SNPs, rs10974944 and rs12343867 are reported in 77% and 85% in association with JAK2 V617F mutation.
Prevalence of JAK2 abnormalities in hematologic neoplasms

JAK2 V617F mutation is reported only in myeloid neoplasms with a high frequency in PV, ET, PMF and refractory anemia with ring sideroblast and thrombocytosis (RARS- t). It is rarely reported in CML, but not in ALL or molecularly characterized eosinophilic neoplasms and mastocytosis, i.e. those with abnormalities in PDGFRα, PDGFRβ, FGFR1 or KIT. JAK2 rearrangements are seen in some cases of AML, atypical CML and ALL, while exon 12 mutations are only reported in PV (Table 1).

Screening and quantification tests

Detecting JAK2 mutations, determination of hetero-or homozygosity, and the allele burden, i.e. the ratio of the mutant allele to the wild-type, are the aims of carrying out molecular studies. Variable screening techniques are available and these are basically utilizing direct screening, allele specific polymerase chain reaction (PCR) analysis or ultra sensitive PCR techniques with variable sensitivities that can detect mutant gene at the levels of 20%, 3% and 0.01%, respectively. However, quantification techniques are preferred in order to estimate the mutant allele burden, to monitor the disease course and effect of therapeutics. Peripheral blood and bone marrow samples, frozen plasma and paraffin-embedded trephine bone marrow biopsies can all be used.

The roles of JAK2 abnormalities in hematologic neoplasms

Confirmation of clonality

The long list of congenital, secondary or reactive causes for cytosis, cytopenia and dysplasia is creating difficulties in labeling cases for neoplastic conditions without ruling them out, a process that requires extensive investigative steps not to mention the time needed to confirm persistence. Confirming clonality can bypass all these issues and can solve the problematic cases in which a neoplastic condition is co-existing with a secondary or congenital cause. Many methods are used to detect clonality but the most dependable is by performing cytogenetic, fluorescence in situ hybridization (FISH) and/or molecular tests. Abnormal immunophenotype and loss of X-linked polymorphism have many limitations in MPNs. It is currently widely accepted that detecting JAK2 mutation, particularly V617F mutation, is a major criterion in diagnosing myeloid neoplasms, especially MPNs. It is now incorporated in the WHO 2008, Nordic 2007, and BCSH 2007 classifications.

Phenotype and lineage determination

JAK2 abnormalities are seen in many types of hematologic neoplasms as mentioned earlier, and also are reported in 70-80% of cases with unexplained hepatic venous thrombosis and Budd-Chiari syndrome.29

The targeted cell or receptor

It is suggested that the phenotype is determined by the ability of the affected stem cells to differentiate into different lineages, and on the receptor affected by the mutation. For example, EPO, TMP, G-CSF or GM-CSF receptors are targeted to give PV, ET, PMF or chronic myelogenous leukemia, respectively. Another example is the identification of homozygous JAK2 V617F mutation in the endothelial cells in the vessels of PV patients with Budd-Chiari syndrome.

Table 1. Prevalence of JAK2 abnormalities in hematologic neoplasms.

<table>
<thead>
<tr>
<th>Neoplasms</th>
<th>JAK2 V617F mutation</th>
<th>Other JAK2 abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polycythemia vera</td>
<td>&gt;95%</td>
<td>4% JAK2 exon12 mutation</td>
</tr>
<tr>
<td>Essential thrombocytopenia</td>
<td>50-60%</td>
<td></td>
</tr>
<tr>
<td>Idiopathic myelofibrosis</td>
<td>40-50%</td>
<td></td>
</tr>
<tr>
<td>Chronic neutrophilic leukemia</td>
<td>20%</td>
<td></td>
</tr>
<tr>
<td>Chronic myelomonocytic leukemia</td>
<td>3-13%</td>
<td></td>
</tr>
<tr>
<td>Juvenile chronic myelomonocytic leukemia</td>
<td>20%</td>
<td></td>
</tr>
<tr>
<td>Atypical chronic myeloid leukemia</td>
<td>20%</td>
<td>JAK2 rearrangement, t(8;9), t(9;12), t(9;22)</td>
</tr>
<tr>
<td>MPN/MDS-U</td>
<td>12-20%</td>
<td></td>
</tr>
<tr>
<td>MPN/MDS (RARS1)</td>
<td>50-70%</td>
<td></td>
</tr>
<tr>
<td>Myelodysplastic syndrome (RAEB-1,5q)</td>
<td>1-7%</td>
<td>JAK2 rearrangement, t(9;12)</td>
</tr>
<tr>
<td>Secondary AML</td>
<td>4%</td>
<td>JAK2 rearrangement, t(8;9)</td>
</tr>
<tr>
<td>AML (M6)</td>
<td>Few reported cases</td>
<td></td>
</tr>
<tr>
<td>AML (M7)</td>
<td>15%</td>
<td>JAK2 T875N</td>
</tr>
<tr>
<td>M astocytosis</td>
<td>0.25%</td>
<td>JAK2 rearrangement, t(8;9), t(9;12)</td>
</tr>
<tr>
<td>Eosinophilic neoplasms</td>
<td>0.2%</td>
<td></td>
</tr>
<tr>
<td>Chronic myelogenous leukemia</td>
<td>Rare reported cases</td>
<td></td>
</tr>
<tr>
<td>ALL</td>
<td>Non</td>
<td>JAK2 rearrangement, t(8;9), t(9;12), JAK2 R683G and less frequently other R883 point mutations</td>
</tr>
<tr>
<td>ALL in Down syndrome patients</td>
<td>Non</td>
<td>JAK2 R683G, IREED del, other point mutations, insertions and deletions</td>
</tr>
</tbody>
</table>

respectively, with a significant difference if compared to wild-type JAK2.34

The targeted cell or receptor

It is suggested that the phenotype is determined by the ability of the affected stem cells to differentiate into different lineages, and on the receptor affected by the mutation. For example, EPO, TMP, G-CSF or GM-CSF receptors are targeted to give PV, ET, PMF or chronic myelogenous leukemia, respectively. Another example is the identification of homozygous JAK2 V617F mutation in the endothelial cells in the vessels of PV patients with Budd-Chiari syndrome.
Genetic background of the patient

Gender is one of the possible determinants, as ET tend to occur in females more than males, while the opposite is true of PV. Iron metabolism is another factor, as iron depletion will lead to ET phenotype rather than PV. The opposite picture will be seen in relation to EPO bioavailability. SNPs are another possible participant as rs10974944 is significantly seen in patients with PV if compared to ET.\textsuperscript{2,10,30}

Dosage effect

This appears to be the most important factor, and it can be explained by different thresholds of JAK2 kinase activity at which variable receptors, cytokines and proteasomes need to react. This is supported by the reported results of homozygous JAK2 V617F mutation occurring in 25-30% PV compared to 2-4% in ET. Few cases are reported that demonstrate dosage dependent phenotype, like those transforming from ET to PV with increasing JAK2 V617F mutation burden, or from PMF to PV with burden reduction after treatment with hydroxyurea (HU).\textsuperscript{4} We have also encountered a case of PMF that transformed to ET after treatment with HU (K Alkhairi, KM Alayed, MK Alabdulaali, unpublished data, 2007) By monitoring allele burden it is found that JAK2 V617F mutation burden is increasing significantly from ET, PV to PMF.\textsuperscript{23,4,44} The levels at which the burden is found seem to correspond to a specific phenotype of 25%, 55%, 50% and 60% for ET, PV, PMF and post ET or PV myelofibrosis, respectively.\textsuperscript{6} Hypermethylation of the SOCS3 promoter was identified in nearly 1/3 of patients with myelofibrosis and in both JAK2 mutation positive and negative cases,\textsuperscript{27} but not in other types of MPNs; this again will strengthen the role of dosage effect.

Pre-JAK2 mutation event

Several findings raise the possibility of a pre-JAK2 mutation event leading or participating in the development of neoplasia: the presence of JAK2 mutation negative MPNs with 25% abnormal cytogenetics, the finding that 50% of JAK2 mutation positive cases are seen with recurrent cytogenetic abnormalities, and more interestingly, that the majority of JAK2 mutation positive cases develop JAK2 mutation negative leukemic blasts when they progress to AML.\textsuperscript{12,13,30,31} Some of the cytogenetic abnormalities are suspected to present more in association with JAK2 mutation to develop a specific phenotype like del 20q in cases with PV.\textsuperscript{4}

Clinical severity and progression

Thrombosis, bleeding, splenomegaly, bone marrow failure and evolution to acute leukemia are the main complications of MPNs. In ET, many studies suggested that JAK2 mutation positive cases have a higher risk of developing venous thrombosis compared to patients with wild-type JAK2. In a systematic literature review, Panayiotis D. Ziakas analyzed 17 studies with 2,905 ET patients, of whom 1,646 (65.7%) patients were JAK2 V617F mutation positive. Thrombotic events occurred in 523 (31.8%) of mutation positive cases and in 255 (20.3%) of patients with wild-type JAK2. Finally, he concluded that JAK2 V617F patients have a 2-fold risk of developing thrombosis (OR 1.84, 95%CI 1.40-2.43).\textsuperscript{4} Presence of inherited thrombophilia can increase the risk of the development of thrombosis in patients <60 years of age with ET and JAK2 V617F mutation from 2.23 (95%CI 1.57-3.18) to 7.66 (95%CI 2.66-22.03).\textsuperscript{4} High mutation dosage are significantly associated with larger spleen size, higher WBC count, higher lactate dehydrogenase (LDH) level and higher risk of developing venous thrombosis or cardiovascular events in patients with ET or PV. However, these findings are not sufficient for chemical cytoreduction intended risk stratification which is still dependent on age, prior thrombotic event and cardiovascular event-related risk factors.\textsuperscript{2,4,30}

It is still debatable whether an increased JAK2 mutation burden is associated with increased risk of evolution to acute leukemia. However, there is good evidence of its association with progression from ET to PV to PMF if evolution to leukemia then occurs, in the majority of cases the leukemic cells will be JAK2 mutation negative as mentioned earlier. The overwhelming blasts will, therefore, reduce the allele mutation burden which might be considered a sign of evolution if it is not related to chemical cytoreductive therapy (Figure 6).\textsuperscript{14}

JAK2 as a therapeutic target

For decades, the treatment options for patients with MPNs was limited to palliation and preventive measures against the development of thrombosis using aspirin, phlebotomy, splenectomy, splenic radiation or the use of steroids, androgens or EPO in patients with PMF.\textsuperscript{4} Since the middle of the 20th century, aims are widening and touching the areas of prevention of leukemic transformation and cure with the use of cytoreductive medications like busulfan, HU and interferon-alpha (INF-α).
together with the trials of stem cell transplantation. But the use of these options always carries the risk of leukemogenesis. So, given the fact that MPNs are very slow progressing disorders and most of the patients are kept under strict control with non-chemical approaches, the use of these options is limited to high-risk patients.

In the last decade, the use of tyrosine kinase inhibitors is expanding in the management of hematologic neoplasms, especially after the successful results of imatinib in CML patients. However, the use of imatinib in MPNs is only achieving limited benefits. It is reported that the use of oral imatinib in PV can reduce the need for phlebotomy and spleen size, while parenteral administration can achieve remission in 22% of cases. Currently, there are many trials of new agents on patients with PMF, including farnesyltransferase, the aurora family of serine/threonine kinases, vascular endothelial growth factor (VEGF) tyrosine kinase, proteasome and fibrogenesis inhibitors, VEGF neutralizing antibodies and GX15-070MS, an antagonist of the BH3-binding groove of the Bcl-2 family.

Many specific JAK2 inhibitors, like INCB018424, TG101209, TG101348, XL019 and TG10134841, are currently in phase I/II trials on advanced stages of MPNs such as PMF, post-ET or PV myelofibrosis. Recent reports from the trials with INCB018424 are showing significant improvement in splenomegaly, constitutional symptoms, control of myeloproliferation and reduction in allele burden. Non-specific JAK2 inhibitors are also in clinical trials. These include: CEP-701 (an FLT3 inhibitor), tipifarnib (a farnesyltransferase inhibitor), ITF2357 (an HDAC inhibitor) and hypomethylating agents. The main drawbacks of JAK2 inhibitors are hematologic toxicity like neutropenia and thrombocytopenia, non-hematologic side effects which are mainly immunological and endocrinological. Beside that, currently used therapeutic options are well tolerated, and pegylated INF-α and parenteral imatinib are reported to achieve 18-22% remission rates, respectively. Another unsuprising issue is the limitation in curing the disease or preventing evolution in the presence of a JAK2 mutation event and JAK2 mutation negative leukemic transformation. The development of resistance in vitro is another worrying issue. For the anticipated toxic effects of JAK2 inhibitors, few agents targeting component downstream of JAK2 are currently investigated like those inhibiting BCL-XL or SOCS1 mimetics.

Conclusions

JAK2 abnormalities are important contributors but not the sole events in the development of hematologic neoplasms particularly MPNs. Its mutations, rearrangements or del are important to confirm clonality, and their type and dosage can help in classifying hematologic neoplasms. Currently, JAK2 abnormalities are mainly utilized in confirming clonality in diagnosing classical BCR/ABL negative myeloproliferative neoplasms; however, we believe that their role will expand to be added in more hematologic neoplasms, to include the type of JAK2 abnormality in the sub-classifications criteria and, possibly, to consider JAK2 mutation burden in the process of classification and in the assessment of transformation.

The JAK2 V617F positive MPNs are more closely related and appear with variable clinical-pathological phenotypes in response to different modifiers. They seem to have a more severe clinical course but there is still not sufficient available data to include this mutation in the risk stratification. JAK2/STAT pathway is an important target for new therapeutics, but more studies are needed to minimize their toxicity.

References

1. Mughal TI, Goldman JM. Chronic myeloproliferative Disorders. 2008; Informa, UK.


Up-front fludarabine impairs stem cell harvest in multiple myeloma: report from an interim analysis of the NMSG 13/03 randomized placebo controlled phase II trial

Hans E. Johnsen,¹ Lene M. Knudsen,² Anne K. Mylén,³ Peter Gimsing,³ Henrik Gregersen,² Niels Abildgaard,² Niels Frost Andersen,¹ Torben Plesner,¹ Annette Vangsted,¹ Torben Mourits-Andersen⁷ on behalf of the Nordic Myeloma Study Group
¹Aalborg Hospital University of Aarhus, ²Department of Hematology, Odense University Hospital; ³Righospitalet, ⁴Aarhus Hospital, University of Aarhus; ⁵Vejle Hospital; ⁶Herlev University Hospital, ⁷Esbjerg Hospital, Denmark

Abstract

The impact of chemotherapy resistant B cells in multiple myeloma (MM) needs to be evaluated by in vivo targeted therapy. Here we report the conclusions from a phase II randomized, placebo controlled trial adding fludarabine to the induction with cyclophosphamide-dexamethasone. Based on an interim toxicity and safety analysis, the trial was stopped following inclusion of 34 of a planned 80 patients due to a reduced number of patients reaching high-dose therapy and reduced the number of patients reaching high-dose therapy and the trial was stopped. Consequently, fludarabine in combination with alkylating therapy impairs stem cell mobilization and standard therapy in young MM patients and should not be administrated up-front.

Introduction

Due to a range of new drugs there has been a continuous progress in the treatment of multiple myeloma (MM). However, only a few patients are considered cured so far, most likely due to the nature of the disease.¹³ Recent data have indicated that the myeloma cell hierarchy includes resistant circulating clonal memory B cells, which differ considerably from the classical end stage plasma cells, infiltrating the bone marrow. The pathophysiological significance of these cells is unknown, but hypothetically they may serve as “sleeping” myeloma stem cells responsible for and “feeding” post-treatment relapse and disease progression.¹⁷ The clinical impact of these cells needs to be evaluated by in vivo targeted therapy. Therefore, we initiated a randomized phase II multicenter trial comparing induction therapy with cyclophosphamide plus dexamethasone with and without fludarabine, a DNA repair inhibitor. Fludarabine, 9-fl-D-arabinofuranosyl-2-fluoroadenine, is an analog to adenosine cytotoxic against dividing and resting cells.¹² In vivo, the combination of a DNA damaging agent, e.g. adriamycin or cyclophosphamide combined with fludarabine is clinically active against B cells in CLL and low-grade follicular lymphomas.¹³ Although it has been documented active against leukemia and lymphoma, only recent data has suggested efficacy in MM. In a previous open phase II pilot study, we have documented that addition of fludarabine to induction therapy is clinically feasible with only minor toxicity. A beneficial clinical outcome was suggested including a reduction of minimal residual disease (MRD) following the addition of fludarabine.¹¹ However, one concern during the trial design discussion was the adverse impact of fludarabine on stem cell harvest experienced in advanced CLL,¹¹ which, however, was not considered in untreated patient treatment.

The main objective of the subsequent NMSG n.13/03 phase II trial was to generate data on toxicity, safety and efficacy by adding fludarabine to standard induction therapy.¹² In the close follow-up of the patient cohort it was decided to perform an interim analysis, which concluded that fludarabine in the experimental arm inhibits stem cell mobilization capacity and reduced the number of patients reaching high-dose therapy and the trial was stopped. Consequently, fludarabine in combination with alkylating agents should not be administrated as up-front therapy, if high-dose therapy supported by autologous transplantation is standard care.

Materials and Methods

Approval and patient eligibility

The scientific protocols were reviewed and approved by the regional ethics committees in Denmark and the Danish Drug Agency (Sagsnr. KA 03103 ms) and all patients gave written informed consent before study entry. All patients were over 18 years of age and were referred to the departments for diagnostic evaluation. Patients under 60 years of age who had Durie-Salmon stage I with at least one bone lesion, II, or III myeloma were eligible. The criteria for exclusion were prior treatment for myeloma, another cancer, abnormal cardiac function, chronic respiratory disease, abnormal liver function or psychiatric disease.

Key words: multiple myeloma, clinical trial, fludarabine.

Acknowledgments: first, we would like to acknowledge the Pharmacy Copenhagen County for assistance in preparing the placebo and blinding procedures. Second the GCP Unit at Aarhus University Hospital for monitoring the study. This study was supported by unrestricted research grants from Schering Nordic AB and Amgen AB to the Nordic Myeloma Study Group and in part by individual grants to HEJ from the Nordic Cancer Union (grant no 56-9350/56-9351 and 56 100 02-9101/9102 95), the Danish Cancer Society (grant no. 945 100-15) the Danish Research Agency (grant n. 99 00 771) and EU 6th FP (grant n. LSHC-CT-2006-037602 to MSCNET coordinator HEJ).

Received for publication: 12 March 2009.
Revision received: 5 July 2009.
Accepted for publication: 21 July 2009.

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Hematology Reviews 2009; 1:e11
doi:10.4081/hr.2009.e11

Trial design

This was a randomized, placebo controlled, single blinded, phase II study evaluating toxicity and safety of fludarabine added to cyclophosphamide and dexamethasone (CyDex) as induction therapy in younger newly diagnosed symptomatic multiple myeloma requiring therapy. The treatment regimen CyDex as standard induction therapy was documented in NMSG trial n.11/01.¹² Patients were randomized at diagnosis either to CyDex + placebo (control Arm A) or CyDex + fludarabine (experimental Arm B).

Treatment procedure

Fludarabine was considered as the only investigational drug in this study administrated in induction phase I.

Phase I

Arm A (conventional arm): CyDex + placebo, two (three) cycles in Phase I: two courses of CyDex: cyclophosphamide 1000 mg/m² IV day 1 and dexamethasone 40 mg/day PO on day 1-4, and 9-12 + placebo PO; repeated once
day 21. The third cycle of CyDex (without placebo) was only given if the phase II treatment could not be initiated within six weeks after the start of CyDex II. Other steroids in equipotent dose could be used instead of dexamethasone.12

Arm B (experimental arm): CyDex plus fludarabine, two (three) cycles in Phase I: two courses of CyDex: cyclophosphamide 1000 mg/m² day I IV and dexamethasone 40 mg/d (or other steroids in equipotent dose) PO on days 1-4, and 9-12, combined with fludarabine 40 mg/m² PO day 1-3 each cycle; repeated once day 21. The third cycle of CyDex (without fludarabine) was only given if the phase II treatment could not be initiated within six weeks after the start of the second CyDex plus fludarabine course.

Common trunk (phases II–IV)

This was as described in previous reports from NMSG.13,14 In brief, the priming and apheresis phase II included cyclophosphamide 2 g/m² given as a single dose intravenously during 60 minutes. Uroprotection with Mesna 160% of the cyclophosphamide dose divided in four doses (before 3, 6 and 9 h after start of cyclophosphamide) and diuresis of at least 2.5 L/m² the following 24 hours. Granulocyte colony-stimulating factor (G-CSF) was initiated day 4 after cyclophosphamide as Neupogen® 5-10 µg/kg daily adjusted to appropriate vial size. Peripheral blood stem cell leukapheresis were performed during mobilization, guided by CD34 blood levels, by harvest of a minimum of 2×10⁸ CD34⁺ cells per kilogram body weight.

Following harvest of a sufficient graft, the patients passed to phase III: high-dose therapy with melphalan 200 mg/m² given as a single dose intravenously, followed by stem cell infusion 48 hours later, and G-CSF (Neupogen® 5 µg/kg daily or Neulasta® 12 mg) one injection from day 4 after graft reinfusion, until the absolute neutrophil count is more than 1.0×10⁹/L for three consecutive days.

The patients were followed as outpatients during phase IV.

Statistical analysis

The proportions of patients with a given characteristic were compared using Fisher’s exact test for binary data. The distributions of continuous quantities were examined to control that they followed Gaussian distributions with good approximation using Kolmogorov-Smirnov’s test supplemented by Q-Q plots.

If they did, either directly or following transformation (square root or logarithmic), the mean values of the two groups were compared using a t-test. If not the two groups were compared using Mann Whitney’s test.

Results and Discussion

Treatment cycles given during induction phase I

All patients in the conventional arm received the scheduled cycles of therapy. However, in the experimental arm this was only the case for 11/17 patients as 6 patients were stopped before or following the first cycle of therapy (Table 1). Three of these patients did not start therapy, suggesting bias from the clinician, for whom the therapy arm was not blinded. Such a bias may be the result from the relative intensive dosage of fludarabine administered in this trial of 40 mg/m² PO for three days in each of two cycles, compared to our previous trial1 where we administered a dose of 25 mg/m² intravenously for five days. Following discussions within NMSG the protocol group selected oral administration over three days attempting to reach an equivalent total dose of fludarabine as used previously. Our previous experience1 administering a total dosage of 125 mg/m² fludarabine over five days was moderate neutropenia, no thrombocytopenia or severe infectious episodes. We observed all 9 of the fludarabine-treated patients responding to treatment with 2 complete remissions and 7 partial remissions, patients responding to treatment with 2 complete remissions and 7 partial remissions, compared to 5 responders (all PR) in the control-arm. Furthermore, the effect on the blood circulating myeloma compartments identified a significant reduction of CD19⁺ B cells and myeloma plasma cells in the fludarabine-arm, concluding that fludarabine therapy in MM was feasible with a potential clinical efficacy. However, in the current trial, unexpected side effects were initially observed which may explain the drop-out of the 6 patients (Table 1) before the end of induction therapy.

Toxicity and adverse events following induction phase I

In accordance with the CTC criteria, no difference in severe toxicity was found. However, analysis of laboratory quantities following the second treatment showed a borderline reduction of blood lymphocytes from mean 1.12 (SD 0.4) to 0.73 (SD 0.6; p=0.055) and an increased plasma creatinine level from mean 57.8 (SD 14.2) to mean 124.2 (SD 28.8; p=0.035). All other variables registered showed no difference including performance score. Many clinical trials in CLL have shown the combination of fludarabine and cyclophosphamide to have tolerability.13,14 however, the observed significant reduction in renal function may be due to latent myeloma specific kidney impairment. All serious adverse events were reported to The Trial Secretariat within one working day of discovery or notification of the event. Initial serious adverse event information and all amendments or addi-

Table 1. Number of treatment cycles during induction therapy (phase I).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo (n) %</th>
<th>Intervention (n) %</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>(0) 0.0</td>
<td>(3) 17.6</td>
<td>0.054</td>
</tr>
</tbody>
</table>

(Mann Whitney test)

| 1 treatment | (0) 0.0 | (3) 17.6 |
| 2 treatments | (15) 88.2 | (9) 52.9 |
| 3 treatments | (2) 11.8 | (2) 11.8 |

Table 2. Fraction of patients subjected to harvest of an autograft (phase II).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo (n) %</th>
<th>Intervention (n) %</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients subjected to at least one harvest</td>
<td>(11) 64.7%</td>
<td>(4) 23.5%</td>
<td>0.037</td>
</tr>
<tr>
<td>Mean total harvest of CD34⁺ cells ×10⁶ ± SD</td>
<td>(11) 736±465</td>
<td>(4) 416±248</td>
<td>0.12</td>
</tr>
<tr>
<td>Mean average number of CD34⁺ cells ×10⁶ per harvest ± SD</td>
<td>(11) 584±501</td>
<td>(4) 251.2 (152.6)</td>
<td>0.071</td>
</tr>
<tr>
<td>Number of apheresis to collect &gt;5×10⁶ CD 34⁺ cells/kg</td>
<td>Placebo (n) %</td>
<td>Intervention (n) %</td>
<td>p</td>
</tr>
<tr>
<td>No harvest session/mobilization failure</td>
<td>(6) 35.3%</td>
<td>(13) 76.5%</td>
<td>0.11</td>
</tr>
<tr>
<td>1 harvest session</td>
<td>(7) 41.2%</td>
<td>(2) 11.8%</td>
<td></td>
</tr>
<tr>
<td>2 harvest sessions</td>
<td>(2) 11.8%</td>
<td>(15) 88.2%</td>
<td></td>
</tr>
<tr>
<td>3 harvest sessions</td>
<td>(2) 11.8%</td>
<td>(2) 11.8%</td>
<td></td>
</tr>
</tbody>
</table>
tions were recorded on the Adverse Event Form. This was reviewed and documented that CMV-reactivating was seen in one patient in the standard arm and 3 patients in the experimental arm.

**Priming for stem cell mobilization and harvest during phase II**

Fifteen of 17 patients and 12/17 were primed with standard care cyclophosphamide and rhG-CSF in arms A and B, respectively. Successful mobilization to reach the level of >10 CD34+ cells per microliter blood triggered leukapheresis and was obtained in 11/17 patients in arm A but only in 4/17 patients in arm B (Table 2). This difference was significant at the interim analysis performed by an independent group of experts and the decision was taken to stop the trial. Comparison of the total and average number of CD34+ cells harvested did not reveal differences in patients actually undergoingapheresis. The situation concerning published data about the adverse impact of fludarabine on stem cell harvest is now clearer. In a survey of advanced CLL from 122 centers of the European Group of Blood and Marrow Transplantation (EBMT), it was concluded that attention should be given to the timing of mobilization with respect to the last dose of fludarabine. This has been further supported by a study of B-CLL after front-line treatment with fludarabine (30 mg/m2 per day) and cyclophosphamide (200 mg/m2 per day) both given orally for five consecutive days in six monthly courses. After evaluation performed two months after the last course, responding patients were considered for PBPC collection. Following conventional rhG-CSF, priming until adequate blood CD34 circulation was achieved and a harvest procedure was initiated successfully in only 12 of the 32 CLL patients.

The present report supports this observation and further adds to our knowledge that as few as one to two series of fludarabine may result in poor mobilization and impair standard therapy. Recently, the stem cell toxicity has been supported by the observation that the risk for sMDS/AML was correlated to the use of fludarabine supported by the observation that the risk for sMDS/AML was correlated to the use of fludarabine.

**Response evaluation following phase II**

Response evaluation performed following phase I-II prior to high-dose therapy revealed 7/13 and 5/7 patients obtained a partial remission. The trend towards more patients achieving CR in the intervention arm observed in our previous phase II study could not be confirmed in this trial (*results not shown*). There was no difference in graft quality, evaluated by time to neutrophil and platelet recovery. One patient died from treatment complication in the experimental arm due to protocol violation: the patient had impaired renal function and received a full dose of fludarabine. This was reported and reviewed by the Danish Drug Agency. Follow-up in December 2008 revealed that 12/13 and 9/13 patients had responded to therapy with 4/13 and 3/13 obtaining CR at any time during follow-up. The number of patients dying from complications or progressive disease was 2/17 and 5/16, respectively, with no significant difference between the two arms.

**Conclusions**

In conclusion, the scheduled fludarabine dosage in two cycles combined with alkylating therapy impairs stem cell mobilization and standard therapy in young MM patients and should not be administrated up-front. This observation is in accordance with recent data from up-front therapy in CLL. We are now left with the challenge of understanding the mechanisms of fludarabine responsible for the negative side effect on mobilization of normal hematopoietic progenitors, as well as the potential therapeutic effect on marrow and blood B cells in MM and other B-cell malignancies. This is of special interest as the myeloma cell hierarchy includes resistant circulating clonal memory B cells, which differ considerably from the classical end stage plasma cells, infiltrating the bone marrow. The pathophysiological significance of these cells is at present unknown, but hypothetically they may serve as “sleeping” myeloma stem cells responsible for and “feeding” post-treatment relapse and disease progression, as studied by the Myeloma Stem Cell Network supported by the Myeloma Platform 6th FP from the EU.

**References**

Maintenance therapy in multiple myeloma

Jean-Luc Harousseau
Rene Gauducheau Cancer Center, Nantes/St. Herblain Cedex, France

Introduction

The treatment of multiple myeloma (MM) has changed dramatically in the past twenty years with the introduction of high-dose therapy plus autologous stem-cell transplantation (ASCT) in younger patients and, more recently, of three novel agents (thalidomide, bortezomib, and lenalidomide).

When conventional chemotherapy was the only available possibility, complete responses (CR) were very rare and the objective of maintenance was to prolong remission duration by continuing the same type of treatment that induced the initial response. With recent therapeutic improvements, CR achievement becomes a realistic goal that, in most cases, is significantly correlated with the outcome. Therefore, both the nature and the impact of maintenance therapy have changed. Maintenance therapy is based currently on novel agents, and its objective is not only to control the clone but also to further decrease the tumor burden and improve the quality of response.

A number of randomized studies show a benefit from maintenance therapy with novel agents (until now, mostly thalidomide), at least in terms of response rate and progression-free survival (PFS). However, there is still a debate as concerns the impact on overall survival (OS) and the optimal administration of maintenance therapy.

Maintenance therapy in the pre-thalidomide era

In patients initially treated with conventional chemotherapy, maintenance chemotherapy has failed to demonstrate any significant benefit. Therefore, the recommendation was to stop chemotherapy once stable response had been achieved (the so-called plateau phase). One study showed a survival advantage for patients receiving alternate-day prednisone maintenance therapy at a dose of 50 mg when compared with 10 mg after standard induction chemotherapy.

In the nineteen-eighties α-interferon maintenance therapy represented a great hope. Following the initial randomized study showing prolonged remissions with α-interferon maintenance in patients responding to conventional induction therapy, a number of randomized trials were performed but their results were controversial. Two meta-analyses of randomized trials showed that with interferon maintenance, time to PFS and OS was increased by four to seven months. However, most investigators considered that the benefit was small and needed balancing against cost and potential toxicity of prolonged treatment with α-interferon.

In addition, α-interferon has been used after ASCT, with the hypothesis that it might be more effective in patients with minimal residual disease. In a retrospective analysis of the European Bone Marrow and Blood Transplant Registry, interferon maintenance was associated with improved PFS and OS in patients responding to high-dose therapy. However, two randomized trials failed to confirm this result.

Thalidomide as maintenance therapy after autologous stem-cell transplantation

When thalidomide maintenance therapy was first introduced in the field of ASCT for MM, the median duration of response did not exceed three years and almost all patients did relapse ultimately. Six randomized trials have evaluated the impact of thalidomide maintenance after ASCT in MM. However, these trials differed in their design and in the dose and duration of thalidomide maintenance (Table 1).

In two of these studies, patients were randomized initially to receive thalidomide throughout their treatment (both before and after ASCT). Therefore, the putative impact of thalidomide was not expected to be a result of the maintenance effect only.

In the other four studies, patients were randomized after ASCT to receive either thalidomide or not. While in two of these trials the control group was to receive no further treatment, in the Tunisian study by Abdelkefi et al. (2008), thalidomide maintenance was compared to a second ASCT, and in the Australian study by Spencer et al. (2009) the combination of thalidomide plus alternate-day prednisone was compared to the administration of prednisone alone.

In two of these trials, patients were randomized only if they were not progressing after induction treatment plus ASCT, while in the other two studies, all patients were randomized, whatever the degree of post-ASCT response. Finally, in the French and Australian studies by Attal et al. (2006) and Spencer et al. (2009), respectively, thalidomide was not used during induction treatment. In the British study by Morgan et al. (2008), there was an initial randomization at diagnosis (thalidomide versus no thalidomide), and in the Tunisian study by Abdelkefi et al. (2008), all patients received thalidomide plus dexamethasone as induction treatment prior to ASCT.

In four of these four trials, thalidomide was prescribed until relapse or until severe adverse event, while in the studies by Abdelkefi et al. (2008) and Spencer et al. (2009), the duration of thalidomide was fixed (six months in the Tunisian study and 12 months in the Australian study). The daily dose of thalidomide varied from 100 mg/day to an initial dose of 400 mg/day in the first two of the six studies. Despite these disparities, all six studies showed a benefit in favor of thalidomide in terms of response rate (CR, or greater than or equal to very good partial remission and PFS) (Table 2).

Results are not that clear-cut as regards OS. While in the initial publication of the French and Tunisian studies OS was significantly longer in the thalidomide trials, with longer follow-up this survival advantage disappeared, and Abdelkefi et al. (2009) did publish a retraction recently.

On the contrary, the first publication from the Arkansas group showed no significant difference in OS between the two groups, because of a shorter OS after relapse in patients initially treated with thalidomide. However, with a longer follow-up, the OS curves diverge after five years. A second report of the same trial, showed a trend in favor of thalidomide and a significant benefit in the subgroup of patients with karyotypic abnormalities. In the Dutch (Lokhorst et al., 2008) and British (Morgan et al., 2008) studies, the PFS benefit did not translate into a significant OS benefit, again because of a shorter OS after relapse in the thalidomide group.
How can we analyze these differences?

The first message is that OS data should not be analyzed and published too early. In the past the only possibility at relapse was conventional chemotherapy. Currently we have more possibilities (e.g., ASCT, thalidomide and lenalidomide, bortezomib), and survival after relapse may be longer than duration of first response. Since it is possible to achieve median OS of more than or equal to five years now, OS should not be analyzed before at least five-year median follow-up time.

Secondly, OS obviously depends on salvage treatments. If thalidomide is prescribed until relapse or severe toxicity, one can imagine that thalidomide should not be used at relapse. Therefore, more patients receive thalidomide at relapse in the no-thalidomide trials, and the design of these studies is rather early (up front) thalidomide versus late (at relapse) thalidomide. This was actually the case with ASCT: in randomized trials comparing ASCT and conventional chemotherapy, ASCT was superior in terms of PFS but not always in terms of OS, partly because patients in the conventional chemotherapy group could receive ASCT at relapse. A randomized study comparing early versus late ASCT showed no significant difference in terms of OS.

Moreover, the risk of thalidomide-induced peripheral neuropathy is clearly related to the cumulative dose, and prolonged exposure carries the risk of severe neuropathy, precluding or limiting the use of bortezomib. Prolonged exposure to thalidomide might select clones resistant not only to thalidomide but also to other agents. We already know that lenalidomide is less effective in patients resistant to other agents. Therefore, lenalidomide was not available except for clinical trials. Therefore there were more therapeutic possibilities (including thalidomide) at relapse in the no-thalidomide group.

These considerations clearly raise the issue of the optimal duration of maintenance therapy. Should it be fixed as in the Tunisian or Australian studies, or unlimited as in the other four studies? The theoretical interest of prolonged maintenance is to further improve the level of tumor burden reduction, hence prolonging PFS but, on the other hand, this benefit might be hampered by reduced salvage possibilities, hence a shorter OS after relapse. A randomized study addressing this question might be extremely useful.

Additional questions

What is the optimal schedule of administration?

In the first two studies by Attal et al. (2006) and Barlogie et al. (2006), the initial daily dosage was high (400 mg) and the duration of...
treatment was unlimited, which explains the high incidence of severe neuropathy (Grade ≥3), as well as the percentage of patients who discontinued treatment because of toxicity.\textsuperscript{12,13} Although no randomized study has compared different schedules of administration, doses of 100 mg or 200 mg/day during 6-12 months appear to be effective and better tolerated.\textsuperscript{21,22}

Is thalidomide maintenance useful for all patients, and are we able to predict patients who will benefit from maintenance therapy?

Unfortunately, there is no clear response to this question. In the French study by Barlogie et al. (2006), patients with del(13) apparently did not benefit from thalidomide maintenance, but at the time of this trial, other abnormalities that are frequently associated with del(13), such as t(14;16) or 17p del, were not routinely studied.\textsuperscript{23} We know now that the negative prognostic impact of del(13) is mostly a result of these two additional abnormalities.\textsuperscript{24} We have no published data on the impact of thalidomide in this subgroup of patients with these poor-risk abnormalities, although in a preliminary report (Lokhorst et al., 2008), thalidomide appeared to perform poorly in patients with del(17p).\textsuperscript{27}

In the updated analysis of the Arkansas study by Adelkei et al. (2009), thalidomide significantly improved OS of patients with cytogenetic abnormalities as defined by conventional karyotyping.\textsuperscript{25} This heterogeneous subgroup of patients generally is considered as poor-risk, since the possibility of studying mitoses is associated with a more proliferative disease.

Finally, in the studies by Attal et al. (2006) and Morgan et al. (2008), only patients who did not achieve at least a very good partial response (VGPR) after transplant benefitted from thalidomide maintenance;\textsuperscript{26} but this was not confirmed by Spencer et al. (2009).\textsuperscript{29}

Does thalidomide act as a maintenance or consolidation therapy?

In all the studies reviewed, the PFS prolongation was associated with a CR or CR/VGPR increase. Moreover, the fact that patients who showed CR after ASCT did not benefit from thalidomide in at least two of the studies could mean that thalidomide might act more by increasing the post-ASCT CR rate than by controlling the residual clone. In other words, post-ASCT thalidomide might be considered as a consolidation therapy, and might be administered with the objective of further decreasing the tumor burden. If this is true, we still have to determine the optimal level of response. Is CR with negative immunofixation the requested level or should we try to obtain higher levels of response (stringent response, immunophenotypic response, or even molecular response)?

This important question should be addressed in future trials. To date, Paiva et al. (2008) have shown that immunophenotypic CR, as assessed by multi-parameter flow cytometry, is associated with a better outcome than CR as defined only by immunofixation.\textsuperscript{30}

Other novel agents as maintenance therapy after autologous stem-cell transplantation

Lenalidomide, which is better tolerated than thalidomide and can be prescribed safely for long periods of time, appears to be an ideal candidate for maintenance therapy. However, this agent is more myelotoxic than thalidomide and the optimal dose of lenalidomide after high-dose therapy is not known. Two large randomized trials from the Intergroupe Francophone du Myelome (IFM) and the Cancer and Acute Leukemia Group B (CALGB) groups have tested lenalidomide as maintenance after ASCT, but results of these studies are not available.

In addition, bortezomib has been evaluated in this setting by Morgan et al. (2008) and Paiva et al. (2008).\textsuperscript{31,32} Since bortezomib is associated with a high incidence of peripheral neuropathy when used on a bi-weekly schedule at a dose of 1.3 mg/m², the issue of the toxicity/fecality ratio is crucial.

If the objective of post-ASCT therapy is to increase the level of response with a consolidation effect further, short-term treatment with combinations of novel agents might be attractive as well. Ladetto et al. (2008) recently showed encouraging results with four courses of consolidation treatment with bortezomib-thalidomide-dexamethasone.\textsuperscript{33} In this study, six out of 24 patients, who were at least in VGPR after ASCT, achieved molecular remissions and none of them had a relapse with a median follow-up of 26 months.

Novel agents as maintenance therapy after allogeneic stem-cell transplantation

Currently, allogeneic stem-cell transplantation (allo-SCT) following a myeloablative conditioning regimen has almost been abandoned in MM because of excessive toxicity.\textsuperscript{34} Reduced-intensity conditioning is associated with reduced transplant-related mortality but with increased relapse rate compared to standard allo-SCT.\textsuperscript{35} In order to decrease the relapse rate, a strategy with tandem ASCT-reduced intensity conditioning allo-SCT is currently proposed. However, relapses remain frequent, especially in the absence of chronic-graft versus host disease.\textsuperscript{36} Therefore, post-transplant immunotherapy with donor-lymphocyte infusions and/or novel agents has been tested with the objective of upgrading the level of response. In a preliminary experience, Kroger et al. (2009) have proposed novel agents (thalidomide, bortezomib, or lenalidomide) to patients who were not in CR after allo-SCT and donor lymphocyte infusions.\textsuperscript{37} They could convert partial remission to CR in 59% of patients and to molecular remissions in 50% of patients.

Thalidomide maintenance therapy after non-intensive induction treatment

In a recent trial Ludwig et al. (2009) evaluated thalidomide plus interferon compared with interferon alone in 135 elderly patients with at least stable disease after induction treatment with either thalidomide dexamethasone or melphalan-prednisone (MP). Although PFS was significantly longer in the thalidomide group (24 months versus 13 months, \(p<0.024\)), OS was similar in both groups.\textsuperscript{38}

Five randomized trials have compared MP and MP plus thalidomide (MPT) as the primary treatment in elderly patients.\textsuperscript{39,40} Although the design of these studies and the inclusion criteria were slightly different, all five studies have shown a benefit of MPT in terms of response rate, and PFS was significantly longer in the MPT groups of four out of five studies (Table 3). However, in only two studies this benefit translated into a significantly longer survival,\textsuperscript{39,40} and in these two studies there was no maintenance while in the other three trials there was a maintenance with thalidomide alone in the MPT groups. In the Italian study by Palumbo et al. (2008), the shorter survival after relapse in the MPT group might have been explained by a lower percentage of patients receiving thalidomide as salvage treatment at relapse.\textsuperscript{41} Since the three studies were not designed to address the question of maintenance, it is not possible to consider that the lack of survival benefit in the MPT group is related to maintenance thalidomide. However, one can conclude from these studies that there is no evidence that maintenance thalidomide is useful in elderly patients initially treated with MPT. Until now, in elderly patients, available data do not show a benefit from maintenance treatment with thalidomide, at least in terms of OS. At least two randomized studies addressing the question of maintenance treatment with lenalidomide in elderly patients are ongoing.
Conclusions

In younger patients, post-ASCT maintenance therapy with thalidomide appears to increase tumor burden reduction further, which translates in prolonged PFS. However, the benefit in terms of OS is not clear and many questions remain regarding the respective role of consolidation versus maintenance, the optimal drug and the optimal schedule of administration and duration of treatment, as well as the characteristics of patients who may benefit from this approach. In elderly patients there is currently no evidence that maintenance treatment improves OS.

References

22. Wang M, Dimopoulos MA, Chen C, et al. Lenalidomide plus dexamethasone is more effective than dexamethasone alone in patients with relapsed or refractory mul-
Transcriptional regulation of the human ALDH1A1 promoter by the oncogenic homeoprotein TLX1/HOX11

Kim L. Rice,1 Mansour Heidari,1 Ross H. Taplin,2 Ursula R. Kees,2 Wayne K. Greene1
1School of Veterinary and Biomedical Sciences, Faculty of Health Sciences, Murdoch University, Australia; 2School of Chemical and Mathematical Sciences, Faculty of Minerals and Energy, Murdoch University, Australia; 3Division of Children’s Leukaemia and Cancer Research, Telethon Institute for Child Health Research, Australia

Abstract

The homeoprotein TLX1, which is essential to spleen organogenesis and oncogenic when aberrantly expressed in immature T cells, functions as a bifunctional transcriptional regulator, being capable of activation or repression depending on cell type and/or promoter context. However, the detailed mechanisms by which it regulates the transcription of target genes such as ALDH1A1 remains to be elucidated. We therefore functionally assessed the ability of TLX1 to regulate ALDH1A1 expression in two hematopoietic cell lines, PER-117 T-leukemic cells and human erythroleukemic (HEL) cells, by use of luciferase reporter and mobility shift assays. We showed that TLX1 physically interacts with the general transcription factor TFIIH via its homeodomain, and identified two activities in respect to TLX1-mediated regulation of the CCAAT box-containing ALDH1A1 promoter. The first involved CCAAT-dependent transcriptional repression via perturbation of GATA factor-containing protein complexes assembled at a non-canonical TATA (GATA) box. A structurally intact homeodomain was essential for repression by TLX1 although direct DNA binding was not required. The second activity, which involved CCAAT-independent transcriptional activation did not require an intact homeodomain, indicating that the activation and repression functions of TLX1 are distinct. These findings confirm ALDH1A1 gene regulation by TLX1 and support an indirect model for TLX1 function, in which protein–protein interactions, rather than DNA binding at specific sites, are crucial for its transcriptional activity.

Introduction

TLX1 (HOX11), TLX2 and TLX3 belong to the ancient NKf family of homeobox genes that includes HEX, LBX1/2, MSX1/2, NKK3-2/BAPX1 and NKK2-S/Csx. TLX1 encodes a transcription factor that, although required during normal embryogenesis,2 was actually discovered as a consequence of its aberrant expression in T-cell acute lymphoblastic leukemia (TALL).3,4 Two distinct TLX1-expression categories have been identified in T-ALL.4 High level TLX1 expression (13%) is typically associated with 10q24 chromosomal abnormalities and confers a favorable prognosis whereas TLX1-low T-ALLs (32%) have an intact 10q24 locus and expression does not impact prognosis.5 Aberrant expression of related TLX3 is additionally found in another 22% of T-ALLs following 5q35 chromosomal rearrangements,6 which highlights the significant role of TLX family members in T-cell oncogenesis.

Confirmation that the gene product of TLX1 is an oncprotein has come from mouse models, which have shown that enforced expression of TLX1 impairs cell differentiation and leads to malignancy.11,12 Current models for the mechanism by which TLX1 promotes leukemia are based on its ability to act indirectly, either by enhancing chromosome instability13,14 or by regulating gene expression through specific protein–protein interactions with key cellular regulatory molecules such as the protein serpine/threonine phosphatases PP1 and PP2A, and the transcriptional coactivator/acetyltrans- ferase, CREB-binding protein (CBP).18-20 Thus, TLX1 may mediate its transforming function by simultaneously inhibiting the phosphatase activity of PP1/PP2A to promote cell cycle progression via upregulation of pathways such as those downstream of E2F and MYC,19 and sequestering CBP at heterochromatin to accomplish a differentiation block.20 TLX1 has also long been suspected to act as a sequence-specific transcription factor2,21 that preferentially binds to the core sequence TAA/GTG in vitro,20,21 However, with the exception of its associations with heterochromatic satellite 2 DNA22 and its own promoter,22 no direct target genes for TLX1 have been convincingly identified. Individual genes suspected to be regulated by TLX1 have been described in various settings, including spleen development (Aldh1a1, Kit),23-25 erythroid differentiation (ALDH1A1, c-Kit, Vegf),23,24 and T-cell leukemia (ALDH1A1, Fhl1, Nra43).24,25,26 Nevertheless, the regulatory role that TLX1 plays in such cases still remains to be determined.

The best characterized TLX1 target gene, ALDH1A1 (aldehyde dehydrogenase 1A1) belongs to a subfamily (class 1A) of ALDH genes whose main biological role is the conversion of the aldehyde form of vitamin A (retinoic acid) into its biologically active form, retinoic acid.27 ALDH1A1 apparently has normal roles in embryonic development,27 and in the renewal/differentiation of hematopoietic stem cells (HSCs),28 where it is known to be highly expressed.27 ALDH1A1 is further implicated in regulating the polarity of HSC differentiation by favoring the development of a myeloid rather than a lymphoid cell fate.29 In agreement with this, ALDH1A1 expression can discriminate between acute myeloid (AML) and acute lymphoid leukemia,29 and while we have demonstrated aberrant ALDH1A1 expression in T-ALL,30 ALDH1A1 is reportedly down-regulated in AML.31 Thus, ALDH1A1, which is regulated by TLX1 in its normal chromosomal context29,28 is of interest due to its associations with leukemia.
with both normal development and leukemogenesis. Here, we explored the molecular mechanism(s) by which TLX1 regulates the ALDH1A1 gene and find that it occurs in a non-DNA binding fashion through protein-protein interactions. We further show that TLX1 interacts directly with the general factor TFII B via its homeodomain, indicating a role for TLX1 in gene regulation via the basal transcriptional machinery.

Materials and Methods

Cell culture and expression plasmids

The PER-117 and ALL-SIL T-cell lines, and erythroleukemic cell line HEL, were cultured as previously described.38 The coding regions of human TLX1 and TFII B were amplified by RT-PCR from ALL-SIL cDNA generated by Thermoscript RT (Invitrogen, Carlsbad, CA, USA) using Pfu Turbo DNA Polymerase (Stratagene, La Jolla, CA, USA) and primers containing an Nhe I restriction site. The resulting products were cloned into the Nhe I site of the pCNeo mammalian expression vector (Promega, Madison, WI, USA) and sequence verified.

Luciferase reporter constructs

The human ALDH1A1 proximal promoter region was amplified by high-fidelity PCR from genomic DNA as previously described.39 For the construction of the -978/+42 construct, a 1020 bp fragment of the ALDH1A1 promoter was amplified using the forward primer 5’-GGCCAGTTCAACATCGACCTGCAAGATA-3’ and reverse primer 5’-GGGCTGAGCTTCCCCTCCTGGAAACACAGTGACTGTCGAT-3’ (introduced Sac I/Nhe I restriction sites in italics). To create the -303/+42, -201/+42, -146/+42 and -91/+42 constructs, various lengths of the ALDH1A1 promoter were amplified using the same reverse primer together with the forward primers 5’-GGGAGCTTCAAAAAATATAACTGGCCTTAGTTG-3’, 5’-GGGAGCTTCCAGGTAACAATTGATGCTGGACACTGG-3’, 5’-GGGAGCTTCAAGGCTTCTGGCAAGCTGGTTG-3’ and 5’-GGGAGCTTCCACTGTTGTTCTACCATCCG-3’, respectively. The -50/-42 and +1/+42 constructs were generated directly by oligonucleotide synthesis. All promoter fragments were directionally cloned into the Sac I/Nhe I sites of the luciferase reporter vector pGL3-Basic (Promega, Madison, WI, USA). Insert identities were confirmed by automated DNA sequencing. The FHL1 -821 promoter construct has been previously described.39

Luciferase and β-galactosidase reporter gene assays

Plasmid DNAs (1 µg/µL), prepared using a purification kit (Plasmid Maxi, QIAGEN, Hilden, Germany), were transiently transfected as previously described.39 In brief, PER-117 or HEL cells (1×10⁶) were co-transfected by electroporation (300 V, 960 µF) with 15 µg of luciferase reporter plasmid (or as negative control, pGL3-Basic) and 5 µg of pSV-β-Gal control plasmid. Cells were harvested 24 h later followed by measurement of luciferase and β-galactosidase activities using the Tropix Dual-Light luminescent reporter gene assay system (Applied Biosystems, Foster City, CA, USA). Transcriptional activity was defined as the ratio of luciferase activity (in relative light units; RLU) from pGL3-Basic derivatives relative to β-galactosidase activity from pSV-β-Gal, which reflected the efficiency of transfection. All experiments were repeated a total of three times on different days.

For measuring the effect of TLX1 on ALDH1A1 promoter activity, transfections were similarly performed with the addition of 15 µg of expression plasmid, either pEF-BOS/TLX1, pEF-BOS/TLX1ΔH3 or pEF-BOS as control.39 In this case, transcriptional activity was defined as the log (base 2) transformation of the ratio of luciferase activity (in relative light units; RLU) from pGL3-Basic derivatives relative to β-galactosidase activity from pSV-β-Gal. Statistical analysis was performed in SPSS 2000 using a mixed effects model with day of experiment as a random effect and luciferase reporter plasmid and expression plasmid as fixed effects. Interactions between contrasts comparing pEF-BOS/TLX1 with pEF-BOS, and contrasts comparing ALDH1A1 constructs with pGL3-Basic, were examined. This revealed the extent to which the effect of adding TLX1 was different for the various promoter constructs compared to pGL3-Basic.

Preparation of nuclear extracts

Cells (1×10⁷) were washed twice with 10 ml of cold PBS, resuspended in 400 µl Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA) with protease inhibitors (100 µg/ml aprotinin, 5 µg/ml leupeptin, 1 µg/ml pepstatin A, 0.5 mM PMSF) and incubated on ice for 30 min. The nuclei were pelleted by centrifugation at 6500 g for 1 min and resuspended in 100 µl Buffer C (20 mM HEPES, pH 7.9, 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20% glycerol) with protease inhibitors. The nuclear suspension was stirred vigorously on ice for 30 min. The sample was centrifuged at 15 000 g for 10 min, and aliquots of the nuclear extract were frozen immediately in liquid nitrogen and stored at -80°C until required. The protein concentration of nuclear extracts was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

Electrophoretic mobility shift assay (EMSA)

Double-stranded EMSA probes were prepared from the following oligonucleotides: ALDH CAT (5’-AGTTTGTCCATCCATCGTATCC-3’), ALDH CATMUT (5’-AGTTTGTCCATCGTATTGGCAG-3’), ALDH GATA (5’-GCCCCTGGACATAAAAAGGCAAACC-3’), ALDH GATA MUB (5’-GCGGGTGCAGCTACAGCAAGACA-3’). Probes (20 pmol) were labeled by incubation (37°C for 30 min) with T4 polynucleotide kinase (15 U, Gibco BRL) and 40 µCi [γ-³²P]-ATP (3000 Ci/mmol) in a volume of 20 µL. The radiolabeled probes were purified using Microspin G-50 columns (Amersham Biosciences) and made double-stranded by annealing with an equimolar amount of complementary oligonucleotide in 1 x annealing buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA) and 6 µg of nuclear extract in a final volume of 15 µL. The samples were incubated at 4°C for 30 min and then analyzed by electrophoresis on 4% native polyacrylamide gels in 0.5 x TBE at 10 V/cm. For competition experiments, an excess of unlabeled competitor oligonucleotide was added to reaction mixtures. Additional competitor oligonucleotides used were those containing TLX1 (5’-TTCATTCCATATTCCATTCCAGGCAGTCGAGTACTGTCGAT-3’) or GATA (5’-GAAGCTTCAAGAAGTTGATGACTGTCGATG-3’) binding sites.

Bandshifts were performed in the presence of anti-TLX1 by adding 2 µl of polyclonal rabbit antiseraum raised against the C-terminus (sc-880, Santa Cruz Biotechnology, Santa Cruz, CA). Normal rabbit serum was used for the no antibody control. Following electrophoresis, the gels were transferred to 3MM paper (Whatman, Maidstone, UK), dried and autoradiographed at -80°C.

Western blotting and immunoprecipitation of TLX1 complexes

Western blotting was performed with 50 µg of nuclear extract electrophoresed through a 12% SDS-PAGE gel and transferred to a Hybond ECL membrane (AP Biotech, Little Chalfont, UK). The membrane was blocked overnight in TBS-Tween buffer (1 mM Tris, 7.5 mM NaCl, 0.05% v/v Tween-20) containing 3.5% (w/v) gelatin and incubated with 1:1200 diluted rabbit anti-TLX1 antibody (sc-880, Santa Cruz Biotechnology) (1 h, RT). The membrane was incubated with HRP-conjugated secondary antibody (1 h, RT) prior to visualization of bands by enhanced chemiluminescence (AP Biotech). For immunoprecipitation, nuclear extract from ALL-SIL cells (5×10⁶) was pre-cleared with the addition of protein A/G agarose (Santa Cruz Biotechnology) for 15 min at 4°C. Pre-cleared extracts were incubat
ed with 5 μg of affinity-purified rabbit anti-TLX1 polyclonal antibody (sc-888; Santa Cruz Biotechnology), or no antibody (as control), for 4 h at 4°C with constant gentle rocking. Immune complexes were bound to protein A/G agarose beads, centrifuged and washed with 1.2 mL of cold IP wash buffer (100 mM Tris-HCl pH 7.4, 1% NP40 and 1% deoxycholic acid) containing 500 mM LiCl (once) and 1.2 mL of IP wash buffer (four times).

MALDI-TOF mass spectrometric analyses

Immunoprecipitated proteins were resolved by SDS-PAGE and visualized by silver staining. Bands were excised directly from gels into 96-well microtiter plates (Titertek, Huntsville, AL, USA), destained, and in-gel trypsin digestions performed according to Shevchenko et al.41 Peptides were extracted with 10 mg/mL α-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% trifluoroacetic acid, and aliquots of 0.5 μL applied directly onto a target plate and allowed to air dry. Tryptic peptide masses were then obtained using a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA). Known trypsin autocleavage peptide masses (842.51, 1045.56 and 2211.1 Da) were used for internal calibration of each spectrum. The peptide masses were used to search the Swiss-Prot and NCBI nr protein databases using the MS-FIT database tool. A protein was considered to be identified if a minimum of five measured peptide masses matched calculated tryptic peptide masses and if the peptides identified by these matches provided at least 20% sequence coverage of the identified protein.

GST-pulldown assay

TLX1 and TFIIB cDNAs in pCIneo were transcribed in vitro with T7 RNA polymerase. The products were labeled with [35S]-methionine (Amerham Biosciences) using the TNT coupled transcription-translation system (Promega). GST-TLX1 fusion proteins were expressed from pGEX-6P-1 as described previously.6 The GST-pulldown assay was performed by incubating 15 μg of GST, GST-TLX1 or GST-TLX1ΔH3 immobilized on glutathione sepharose beads (20 μL) with 5 μL of proteins translated in vitro and labeled with [35S]-methionine in 500 μL of binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, 1 mM DTT, 0.5 mM PMSF, 0.05% BSA) for 18-20 h at 4°C with continuous rotation. Bound proteins were washed three times with 500 μL of cold binding buffer and eluted in SDS sample buffer. The eluted proteins were resolved on 12% SDS-PAGE gels and visualized by autoradiography.

Results

Functional analysis of the human ALDH1A1 promoter

Ectopic expression of TLX1 was previously shown to modulate endogenous ALDH1A1 expression.15,38-39 To pursue the molecular mechanism(s) underlying this regulatory effect, we cloned 1020 bp of the human ALDH1A1 5'-flanking region into the pG3L-Basic luciferase reporter vector. This sequence comprised nucleotides -978 to +42 relative to the transcriptional start site (Figure 1). Among the conserved promoter elements identified were a TATA-like sequence (GATA box) at -33 and a single CCAAT box at -74, which has previously been shown to be functionally important for ALDH1A1 expression.40 Transient transfection of a series of deletion constructs (-978/+42, -303/+42, -201/+42, -146/+42, -91/+42, -50/+42 and +1/+42; Figure 1) into PER-117 and HEL cells (which both lack TLX1 expression) showed that the ALDH1A1 promoter was functional in both cell lines and that the sequences between -50 and -91 and between -50 and -146 were required for maximal promoter activity in PER-117 and HEL cells, respectively (Figure 2A). Consistent with previous results in Hep3B liver cells,44 deletion of the CCAAT box at -74 caused a dramatic reduction in promoter activity, suggesting that it is a key element for ALDH1A1 expression in multiple cell types.

Regulation of the ALDH1A1 promoter by TLX1

To determine the effect of TLX1 on the transcriptional activity of the ALDH1A1 promoter, transactivation assays were carried out in PER-117 and HEL cells co-transfected with an expression plasmid containing TLX1 or empty vector (pEF-BOS) as a negative control. The results were expressed as a ratio of the normalized transcriptional activity of each of the two promoter constructs with and without TLX1 (Figure 2B). Western blot analysis confirmed that TLX1 protein was expressed in the transfected cells (Figure 2C). Consistent with our previous findings,15,29,38 the effect of TLX1

Figure 1. Nucleotide sequence alignment of the human and mouse ALDH1A1 promoters. Conserved nucleotides are marked with an asterisk. Numbers at right indicate nucleotide positions of the human sequence relative to the transcriptional start site (bent arrow). Potential regulatory elements, including a conserved TLX1 site at -257, are boxed and numbered. Solid triangles with numbers demarcate the 5’ ends of the promoter deletion constructs used in the luciferase reporter assays. The two regions used for gel shift analyses are underlined at -85 to -60 and -42 to -19. BRE, TFIIB recognition element.
on ALDH1A1 was reversed in the transcriptional assay as compared to regulation of the endogenous gene. In PER-117 cells, TLX1 repressed ALDH1A1 promoter activity (Figure 2B), and this required the sequence between -50 and -91, which contains the CCAAT box. Deletion of the CCAAT box (-91ΔCAT) completely abolished TLX1-mediated repression (Figure 2B), suggesting that downregulation may possibly occur via this site. Interestingly, the shortest constructs (-50/+42 and +1/+42) were mildly stimulated by TLX1 in both PER-117 and HEL cells (Figure 2B) indicating the presence of a separate, positive-acting TLX1-response sequence localized to the region between +1 and +42. No effect by TLX1 was observed on the region between -201 and -303, which contains a conserved TLX1 binding site at -257 (Figure 1), suggesting that TLX1 binds to a distinct recognition sequence in the ALDH1A1 promoter in vitro, or alternatively, that it acts via a non-DNA binding mechanism. In either case, these data indicate that TLX1 operates via at least two mechanisms in respect to the ALDH1A1 promoter; a general transactivating activity via an element located between +1 and +42 and a strong, cell line-specific repressive activity via an element located between -91 and -50.

The homeodomain is required for TLX1-mediated repression

To assess whether the homeodomain of TLX1 is required for the transcriptional activities of TLX1, a mutant TLX1 expression vector (TLX1ΔH3) was employed, which lacks the DNA recognition helix (helix 3) of the homeodomain. Whereas, TLX1 repressed the activity of the basal ALDH1A1 promoter (-91/+42) 4-fold in PER-117 cells, TLX1ΔH3 not only lacked the ability to negatively regulate ALDH1A1, but switched to become an activator, stimulating transcription by approximately 5-fold (Figure 3). A similar result was obtained when the promoter of another target gene, FHL1, was used (Figure 3). By contrast, the positive regulation of the -91/+42 construct observed in HEL cells was comparable when either TLX1 or TLX1ΔH3 was used (5.5- and 8-fold induction, respectively). Thus, the homeodomain is crucial for TLX1-mediated repression but is not required for TLX1-mediated activation, indicating that these two activities are distinct.

TLX1 does not affect formation of transcriptional complexes at the -74 CCAAT site

We opted to focus on the mechanism by which TLX1 mediates CCAAT-dependent repression. Mobility shift assays were therefore performed to identify whether TLX1 directly binds to the CCAAT box or interferes with the binding of other factors at this site. Nuclear extracts derived from PER-117 or HEL cells, either expressing empty pEF-BOS (as control) or pEF-BOS/TLX1, were incubated with a radiolabeled probe (ALDH CAT) spanning the CCAAT motif (-85 to -60; Figure 1). As shown in Figure 4A, incubation of nuclear extract from PER-117 or HEL cells with ALDH CAT resulted in the appearance of at least two specific DNA-protein complexes, together with a third non-specific complex (C2). The strongest, complex C1, appeared to be common to the two cell types, while additional complexes P and H were unique to PER-117 and HEL cells, respectively. These complexes were inhibited by the addition of a 35-fold molar excess of unlabeled self-competitor (ALDH CAT) but not by a 35-fold molar excess of CCAAT mutant competitor (ALDH CATMut; CCAAT to GACTG). Contrary to expectation, in both PER-117 and HEL cells the mobility shift pattern was identical regardless of TLX1
expression status (or addition of TLX1 antibody; data not shown), suggesting that TLX1 does not affect the formation of DNA-protein complexes at the CCAAT box.

**TLX1 alters DNA-protein complex formation at the -33 GATA box**

TLX1 may directly or indirectly inhibit CCAAT-dependent transcription via the basal apparatus. The *ALDH1A1* promoter lacks a canonical TATA box but does possess a related GATA box (GATAAA) utilized by a number of genes whose transcriptional initiation involves interplay between TFIID and GATA factors.44-48 EMSAs were thus performed to identify whether TLX1 directly binds to the GATA box or interferes with the binding of other factors at this site. Nuclear extracts derived from PER-117 or HEL cells, either expressing empty pEF-BOS (as control) or pEF-BOS/TLX1, were incubated with a radiolabeled probe (ALDH GATAB) spanning the GATA box (-42 to -19; Figure 1). As shown in Figure 4B, incubation of nuclear extract from PER-117 cells lacking TLX1 expression with ALDH GATAB resulted in the appearance of four specific DNA-protein complexes, C1, C2, P1 and P2. HEL nuclear extracts only produced three specific complexes (C1, C2 and H), the strongest of which (C2) appeared to migrate similarly in both cell types. These complexes were inhibited by the addition of a 35-fold molar excess of unlabeled self-competitor (ALDH GATAB) but not by a 35-fold molar excess of GATA box mutant competitor (ALDH GATABMut; GATAAA to CTCAGC). Strikingly, in PER-117 but not HEL cells, expression of TLX1 resulted in a significant alteration in complex formation. Of the four specific PER-117 complexes, the formation of two (C1 and P1) was completely inhibited by TLX1, while the intensity of the remaining two (C2 and P2) was enhanced. Thus, TLX1-mediated regulation of *ALDH1A1* in T cells, but not erythroid cells, is associated with an alteration of transcription factor binding at the GATA box. Complex formation at the GATA box in both PER-117 and HEL cells was also strongly inhibited when a 35-fold molar excess of an unlabeled consensus GATA oligonucleotide (GATA; containing WGTAT with different flanking sequences to ALDH GATAB) was used (Figure 5A). This indicated that GATA factors are present in all complexes formed in this assay. However, TLX1 does not appear to bind DNA at this site, since a 35-fold molar excess of an unlabeled consensus satellite 2 oligonucleotide (TLX1), capable of being bound by TLX1,46 could not compete for complex formation (Figure 5A). In agreement with this conclusion, no supershift or inhibition of complex formation was observed following addition of TLX1 antibody (Figure 5B). Notably, however, PER-117 complex C1, which is abolished by TLX1, re-formed in the presence of TLX1 antibody, indicating that TLX1 directly contributes to the disruption of this low mobility complex.

**TLX1 interacts with TFIIB**

To help understand the transcriptional regulatory function of TLX1, we searched for binding partners using an immunoprecipitation strategy in leukemic T cells (ALL-SIL) that

![Figure 4. Effect of TLX1 on ALDH1A1 promoter DNA-binding complexes.](image-url)
aberrantly express TLX1 as a consequence of a 10q24 chromosomal translocation. A TLX1 antibody was employed to isolate naturally occurring nuclear protein complexes, which were separated by SDS-PAGE and silver stained (Figure 6A). Excised gel slices representing discrete molecular mass intervals were digested with trypsin and analyzed by MALDI-TOF mass spectrometry in order to determine the identity of the protein bands. Table 1 summarizes the eight proteins detected. Among these were the centromeric proteins CENP-E and CENP-F, an intriguing finding given that TLX1 has previously been localized to centromeric regions. Of particular interest was the detection of the general transcription factor TFIIB, which was identified by the presence of 15 peptides with sequence coverage of 49% (Table 1). Of particular interest was the detection of the general transcription factor TFIIB, which was identified by the presence of 15 peptides with sequence coverage of 49% (Table 1). To confirm that TLX1 could physically interact with TFIIB in a specific manner, we performed a GST pulldown assay (Figure 6B). Glutathione-Sepharose beads containing GST-TLX1, GST-TLX1ΔH3, or GST alone were incubated with in vitro translated 35S-labeled TFIIB or TLX1 protein. The latter was included as a positive control since TLX1 has previously been shown to homodimerize. Following extensive washing, retention of TFIIB was observed with GST-TLX1 but not with control GST beads (Figure 6B), demonstrating that TLX1 has the capacity to interact with TFIIB. Interestingly, retention of TFIIB, but not TLX1 control, was greatly diminished when GST-TLX1ΔH3 beads were used, indicating that helix 3 of the TLX1 homeodomain contributes strongly to the TFIIB interaction.

**Discussion**

TLX1 has previously been characterized as a DNA-binding factor that preferentially associates with the core sequence TAAT/GTG in vitro and the similar sequence T/GATTC present in satellite 2 DNA. In addition, TLX1 can switch, in a cell type- and promoter context-dependent manner, between roles as activator and repressor; however the mechanism(s) responsible for these divergent roles is poorly understood. In this study, we further investigated the function of TLX1 by analyzing its ability to transcriptionally regulate ALDH1A1, a gene previously identified as being TLX1-dependent in developing mouse spleen as well as in several cell lines (PER-117, HEL, NIH-3T3), and which contains a predicted TLX1 binding site, conserved between human and mouse, at -257 upstream of the transcriptional start site. In the first instance, our data confirmed ALDH1A1 as a regulatory target of TLX1, with the polarity of effect observed in terms of activation/repression being heavily dependent upon cell type. In PER-117 T cells, transient luciferase reporter assays with nested deletions of the ALDH1A1 promoter revealed that TLX1-mediated repression occurred in a CCAAT box-dependent manner involving an element located between -91 and -50. By contrast, TLX1 stimulated transcription in a CCAAT-independent manner from a proximal location (-91/+42) in human cell lines. The migration of the free probe is indicated along with the positions of specific DNA-protein complexes (C1, C2, P1 and P2 in PER-117 cells; C1, C2 and H in HEL cells). Overbars denote the addition of a 35-fold molar excess of unlabelled double-stranded self (ALDH GATAB), satellite 2 DNA (TLX1) or GATA consensus (GATA) probe as competitor. Asterisks denote complexes altered by TLX1. (B) TLX1 directly disrupts the low mobility ALDH1A1 promoter GATA box complex C1. EMSA performed as above using the -33 GATAAA site oligonucleotide (ALDH GATAB) in the absence (-) or presence (+) of TLX1 antibody. The migration of the free probe is indicated along with the positions of specific DNA-protein complexes (C1, C2, P1 and P2 in PER-117 cells; C1, C2 and H in HEL cells). Overbars denote the addition of a 35-fold molar excess of unlabelled double-stranded self (ALDH GATAB), satellite 2 DNA (TLX1) or GATA consensus (GATA) probe as competitor. Asterisks denote complexes altered by TLX1. (B) TLX1 directly disrupts the low mobility ALDH1A1 promoter GATA box complex C1. EMSA performed as above using the -33 GATAAA site oligonucleotide (ALDH GATAB) in the absence (-) or presence (+) of TLX1 antibody. The migration of the free probe is indicated along with the positions of specific DNA-protein complexes (C1, C2, P1 and P2 in PER-117 cells; C1, C2 and H in HEL cells). Overbars denote the addition of a 35-fold molar excess of unlabelled double-stranded self (ALDH GATAB), satellite 2 DNA (TLX1) or GATA consensus (GATA) probe as competitor. Asterisks denote complexes altered by TLX1. (B) TLX1 directly disrupts the low mobility ALDH1A1 promoter GATA box complex C1. EMSA performed as above using the -33 GATAAA site oligonucleotide (ALDH GATAB) in the absence (-) or presence (+) of TLX1 antibody. The migration of the free probe is indicated along with the positions of specific DNA-protein complexes (C1, C2, P1 and P2 in PER-117 cells; C1, C2 and H in HEL cells).
erythroleukemic (HEL) cells. Transactivation, which was also observed in the T-cell background when a minimal promoter sequence was used (-50/+42), was abolished in HEL cells with the addition of extra DNA sequence (-146/-92) suggesting that a specific factor bound to this region is capable of abrogating the stimulatory potential of TLX1. Thus, TLX1 possessed two activities in respect to the ALDH1A1 promoter, namely CCAAT-dependent repression, which was cell type-specific and cryptic CCAAT-independent activation, which was unmasked by deleting upstream regulatory sequences. Neither of these activities involved the TLX1 recognition site at -257, which initially suggested that TLX1 either bound a distinct sequence in vivo, as is the case with the regulation of its own promoter,25 or that it acted via a non-DNA binding mechanism.

Remarkably, the effect of TLX1 on ALDH1A1 promoter activity in the transient reporter assays was inversely related to its previously observed effect on endogenous ALDH1A1 levels in PER-117 and HEL cells. This phenomenon has been observed previously in respect to other putative gene targets of TLX1, namely NR4A3, KIT and FHL1.15,25,29 Although puzzling, the finding that a homeoprotein can act as a repressor or activator of transcription depending on promoter context is a common one. In many cases the activity that predominates has been found to be highly dependent on the nature of the cis-regulatory DNA sequence since this can, in turn, affect the interaction of homeoproteins with co-regulatory molecules such as TALE (three amino acid loop extension) homeoproteins, CBP (CREB-binding protein) or Groucho.50-54

CCAAT-dependent repression by TLX1 was found to require the DNA recognition helix (helix 3) of the homeodomain. Intriguingly, the TLX1ΔH3 mutant lacking this helix was not only incapable of repressing ALDH1A1 transcription in PER-117 cells but switched to become an activator of transcription. This indicated that the repression/activation activities of TLX1 are separable with a structurally intact homeodomain being absolutely required for transcriptional repression, but not activation. Given these findings, a reasonable assumption was that TLX1 would repress ALDH1A1 transcription by directly binding promoter DNA at or near the CCAAT box. This crucial element is likely generally required for ALDH1A1 expression, since it was also identified as the major positive element in the Hep3B cell line and in Hepa-1 mouse hepatoma cells where it was bound by NF-Y and CCAAT/enhancer binding protein β (C/EBPβ), respectively.6,25 Moreover, the CCAAT box is capable of being bound by the CCAAT binding transcription factor (CTF1/NFIC) with which TLX1 is known to interact in a functional manner.6,24 However, EMSA assays using PER-117 (or HEL) nuclear extracts revealed that TLX1 did not directly

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Figure 6. TLX1 interacts with TFIIB. (A) Isolation of TLX1-associated proteins. Nuclear extracts from ALL-SIL leukemic T cells were immunoprecipitated using TLX1-specific antibody (Ab), or no antibody (No Ab) as negative control. Coprecipitated proteins were resolved by SDS-PAGE and silver stained. (B) GST pulldown assay. GST, GST-TLX1 and GST-TLX1ΔH3 immobilized on glutathione-Sepharose beads were incubated with in vitro-translated, 35S-labeled TLX1 as positive control, and TFIIB. Bound proteins were washed, eluted, resolved by 12% SDS-PAGE and detected by autoradiography: 50% of the labeled input proteins TLX1 and TFIIB are shown on the left.
Indeed, we found that TLX1 may abrogate CCAAT-dependent transcription of *ALDH1A1* by acting through the basal transcriptional apparatus. Supporting this notion, a previous study demonstrated that TLX1 is capable of repressing transcription via the RNA polymerase II holoenzyme in a manner that is DNA-independent, yet requires helix 3 of the homeodomain. Indeed, we found that TLX1 altered the formation of GATA-containing protein complexes at the non-canonical TATA (GATA) box located at -33 on the *ALDH1A1* promoter and that this occurred specifically in PER-117 cells where repression by TLX1 was observed. The GATA box is a dual regulatory site capable of binding both GATA factors and members of the basal transcriptional machinery, which is over-represented among erythroid-specific gene promoters. Studies to date have indicated that the GATA box is a specialized/weakened TATA box whose activity depends on interplay between binding of GATA factors and general transcription factors such as TFIIID. Based on our data, we therefore postulate that TLX1 can operate as a transcriptional repressor by altering the balance between specific factors binding at the GATA box without actually binding DNA itself. This, at least in part, may involve direct protein contacts by TLX1 since inclusion of anti-TLX1 antibody into EMSA binding reactions reduced TLX1-associated inhibition of one of the GATA box complexes identified. Intriguingly, the related HEX protein has been reported to interact with GATA2 to inhibit its binding to the *Ifk-1/KDR* gene, suggesting that direct antagonism of GATA factors may be a feature of NKI homeoprotein function more generally.

We further showed that TLX1 specifically interacts with TFIIH, a member of the general transcriptional machinery. TFIIH, which can bind promoter DNA in a sequence-specific manner at a TFIIH recognition element (BRE), plays a central role in pre-initiation complex (PIC) assembly, providing a bridge between promoter-bound TFIIH and RNA polymerase II. This points to a mechanism to account for how TLX1 can inhibit basal transcription in a non-DNA binding manner, both in this study and that of Owens et al. Specifically, TLX1 may directly affect repression of the *ALDH1A1* promoter, which contains a BRE adjacent to the GATA box, by inhibiting the rate/extent of PIC formation via contacts with TFIIH. Notably, the TLX1-TFIIH interaction was reduced in the absence of homeodomain helix 3. Thus our results provide one potential mechanism to explain the switch in activity from repression to activation observed with the TLX1ΔH3 mutant.

Significantly, it has been recently reported that TLX1, like many other homeoproteins, can form a mutually inhibitory complex with the co-activator/histone acetyltransferase molecule CBP. This interaction leads to sequestration of CBP at repressive chromatin domains, which is consistent with our previous finding that TLX1 can localize to heterochromatin via binding at satellite 2 DNA. The TLX1-CBP interaction was also shown to require helix 3 of the TLX1 homeodomain, thereby suggesting an alternative, although not mutually exclusive, explanation for TLX1-mediated repression and its reversal in the TLX1ΔH3 mutant. Riz et al. further showed a correlation between the presence of TLX1 and a lack of CBP-associated acetylation of GATA1. As is the case for GATA2 and GATA3, the DNA binding and transcriptional activity of GATA1 is heavily dependent on its acetylation status. Taken together, we suggest a model in which interactions between TLX1 and both TFIIH and CBP lead to the transcriptional repression observed in our transient luciferase assays. Whereas contacts with TFIIH may inhibit the formation of a functional PIC, binding to CBP may prevent it functioning as a co-activator and to acetylate GATA factors. This would explain the substantially altered formation of GATA-containing protein complexes observed at the *ALDH1A1* promoter GATA box, despite the lack of DNA binding by TLX1. It is conceivable that a general ability of TLX1 to indirectly regulate target genes by altering GATA factor activity, whether via CBP or an alternative mechanism, may be consequential for its role as an oncoprotein. This is particularly in view of evidence linking both a blockage in T-cell development and leukemogenesis to incapacitated GATA function. Interestingly, a non-DNA binding mode of action for TLX1 that involves activity regulation of other transcription factors is reminiscent of another important T-ALL oncoprotein, SCL/TAL1, which induces leukemia by recruiting the co-repressor/histone deacetylase mSin3A to inhibit the transcriptional activity of E47/HEB.

There is little evidence to date showing that TLX1 can bind directly to natural DNA sequences to regulate target gene expression. Instead, TLX1 has been shown to operate indirectly by interacting with other proteins, most notably the phosphatases PPI and PP2A to regulate gene cascades in various pathways such as RB/E2F and pi07/MYC. Consistent with this paradigm, our data confirm that TLX1 is capable of regulating *ALDH1A1* expression in a non-DNA binding manner by affecting transcriptional complexes at the proximal *ALDH1A1* promoter, although clearly, additional cis-regulatory elements (which may influence TLX1 protein interactions) are required to recapitulate the effect of TLX1 on endogenous *ALDH1A1* gene expression. We showed that TLX1, like other homeodomain transcription factors including tinman/Nkx2-5, abdominal-A, Nkx6.1 and Vnd/Nkx2.2, is capable of acting as a bi-functional transcriptional regulator, whose activation and repression activities operate in a cell-type specific manner and via two distinct mechanisms. The first involves the ability of TLX1 to repress transcription, possibly by perturbing interactions between CCAAT box-binding transcriptional activators and proteins (GATA factors/basal transcriptional machinery) assembled at a non-canonical TATA (GATA) box. This activity does not appear to involve direct DNA binding, although a structurally intact homeodomain is required, presumably in order for TLX1 to interact with TFIIH and/or CBP. Chromatin immunoprecipitation assays to identify the specific transcription factor(s) bound at this composite CCAAT-box/TLX1 responsive element and at the GATA box before and after TLX1 expression are required to substantiate this hypothesis. The second activity involves the ability of TLX1 to stimulate transcription through as yet unidentified regulatory elements in the proximal *ALDH1A1* promoter and does not require an intact homeodomain. Our understanding of the role of TLX1 in normal development and in T-cell leukemogenesis is crucially dependent on deciphering the mechanisms by which TLX1 is capable of regulating gene expression. Future work to characterize TLX1 target genes and to fully define the protein participants involved in TLX1-mediated gene regulation will represent important steps towards this goal.

References

homeobox gene lies at the chromosome 10 breakpoint in lymphoid neoplasias with chromosomal translocation t(10;14). Blood 1991;78:2996-3003.


46. Aird WC, Parvin JD, Sharp PA, Rosenberg RD. The interaction of GATA-binding proteins and basal transcription factors with GATA box-containing core promoters. A model of tissue-specific gene expression. J


Communication between bone marrow niches in normal bone marrow function and during hemopathies progression

Sara Lamorte,1 Leonor Remédio,1 Sergio Dias1,2,3
1Centro de Investigação de Patobiologia Molecular, CIPM, Instituto Português de Oncologia de Lisboa Francisco Gentil, E.P.E.; 2CEDOC, Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Lisboa, Portugal; 3Instituto Gulbenkian Ciência, Oeiras, Portugal

Abstract

Hematopoietic stem cell (HSC) chemotaxis, adhesion, proliferation, quiescence and differentiation are regulated by interactions with bone marrow (BM) niches. Two niches have been identified in the adult BM: the endosteal (close to the bone) and the perivascular niche (close to blood vessels). A vast body of literature has revealed the molecular basis for the interaction of HSCs with the two niches. However, the signals that regulate the communication between the two niches have not been well defined. Taking in consideration several clinical and experimental arguments this review highlights the molecular cues, involved in the communication between the BM niches, which regulate the basic properties of HSCs in physiological and malignant conditions. As such, it aims at clarifying the most important advances in basic and clinical research focusing on the role of different factors in the regulation of the BM microenvironment.

Introduction

Hematopoietic stem cells reside in bone marrow niches, which regulate their fate

Hematopoietic stem cells (HSCs) are self-renewing cells which give rise to all types of mature blood cells. HSCs can be subdivided into long-term HSCs (LT-HSCs) and in short-term (ST-HSCs). LT-HSCs can give rise to all blood lineages and have unlimited self-renewal capacity. ST-HSCs produce ST-HSCs which are still multipotent but with limited self-renewal capacity. ST-HSCs differentiate further into lineage-committed progenitor cells which are responsible for the large-scale production of mature blood cells.1

The bone marrow (BM) is the major site of adult hemopoiesis, but, in pathological conditions, hemopoiesis can also occur in extra-medullary sites like thymus, spleen and liver. HSCs are localized in specialized microenvironments within hematopoietic tissues called niches.2,4 Within the BM, two anatomical and functional niches have been proposed, the endosteal niche5,6 and the perivascular niche.11 It has been suggested that about 60% of bone-marrow HSCs are adjacent to perivascular niches and up to 20% of HSCs localize in the endosteal niches; the remaining HSCs are believed to be scattered throughout the BM.10,12 Endosteal niches, located at the inner bone surface, contain quiescent HSCs, characterized by a low proliferative rate; whereas activated HSCs, which undergo differentiation and ultimately mobilization to the peripheral circulation, are in close contact to sinusoids of the BM microvasculature in the perivascular niche.8,10 Endosteal niches may thus represent a reserve of HSCs, while perivascular niches connect HSCs to the blood stream.

The endosteal niche mainly comprises endosteal cells, osteoblasts and osteoclasts, while the perivascular niche contains mainly endothelial cells. Stromal cells, including reticular and mesenchymal cells, are common components of both niches. They are scattered throughout the trabecular space of the BM and surround the endothelial cells. As these cells are a component of both endosteal and vascular niches, they may serve as a cellular link between them.13 The cellular components of the niches interact with each other to support HSC adhesion, quiescence, chemotaxis and, in the case of the vascular niche, differentiation.10,11,14,17,32 Thus, the BM niches are a component of their physiological and functional responses depend on specific interaction with BM niches (Table 1).

Chemotaxis

Bone marrow niches recruit hematopoietic stem cells

HSC chemotaxis towards the endosteal niche has been suggested to be mediated by osteopontin (Opn) and calcium ion concentration ([Ca2+]o).5 Opn, a glycoprotein expressed on endosteal bone surface by osteoblasts, promotes HSC migration, as shown in vitro studies with Opn-/- mice. In these mice, there is a migration defect of HSCs, which undergo differentiation and ultimately mobilization to the peripheral circulation, are in close contact to sinusoids of the BM microvasculature in the perivascular niche.8,10,11 Endosteal niches may thus represent a reserve of HSCs, while perivascular niches connect HSCs to the blood stream.

Migration of HSCs from the endosteal to the perivascular niche is regulated by c-kit/Scf, chemokine receptor 4 (CXCR4)/stromal-cell derived factor-1 (SDF-1) and granulocyte colony-stimulating factor (G-CSF), pathways.2,10,14,16,43 Endothelial cells and reticular cells have been shown to produce SDF-1, generating a gradient from the perivascular to the endothelial niche, which may thus promote HSCs migration, since CXCR4 is expressed on HSCs.23,28 Mobilization of HSCs from the endosteal to the vascular niche is essential for hematopoietic recovery following myeloablation. In this case, the soluble form of membrane stem cell factor (sSCF), released from osteoblasts after cleavage by SDF-1-induced matrix metalloproteinase-9, promotes HSC homing to the perivascular niche by interacting with its receptor c-Kit.12,17 G-CSF, produced by osteoblasts, promotes the mobilization of HSCs into the peripheral blood by up-regulating CXCR4 expression on HSCs and decreasing SDF-1 expression in the BM. G-CSF, in fact, induces the expression of proteolytic enzymes such as elastase, cathepsin G, MMP-2, and MMP-9, which cleave SDF-1.

Adhesion

Bone marrow niche promotes hematopoietic stem cells adhesion

HSC adhesion to the endothelial cells is mediated by different molecular interactions including N-cadherin/β1-catenin; Tie-2/Angio-
The asymmetric distribution of N-cadherin/β-catenin on the cell surface of HSCs and osteoblasts, respectively, and, in particular, the localization of these molecules at the site of interaction of LT-HSC with spindleshaped N-cadherin/CD45 osteoblastic (SNO) cells, suggested a role for N-cadherin/β-catenin in HSCs adhesion on the endosteal niches. Studies performed by Kiel and collaborators failed to show significant numbers of N-cadherin expressing HSCs, questioning whether HSC adhesion to osteoblasts is mediated by N-cadherin.

Tie2, a receptor tyrosine kinase expressed by a small fraction of BM cells highly enriched for HSC activity in adult murine BM, binds its ligand, Ang-1, expressed by osteoblasts at the surface of trabecular bone. Regarding Opn, its expression is restricted to the endosteal bone surface and contributes to HSCs adhesion to the endosteal region via β1 integrin expressed by HSC. Osteoblasts also express high levels of Anxa2, a calcium-dependent phospholipid-binding protein, and it has been shown, both in vitro and in vivo, that Anxa2 regulates HSCs homing and binding to the endosteal niche, through the binding to its ligand and Anxa2r.

Adhesion of HSCs to the perivascular niche is mediated by cβ1 integrin/vascular cell adhesion molecule 1 (VCAM1) and CD47/E-selectin interaction. cβ1 integrins, expressed by HSCs, interact with VCAM-1, constitutively expressed on BM endothelial cells. Since inactivation of E-selectin and α4 integrin reduces drastically hematopoietic progenitor and stem cell (HPSG) homing into lethally irradiated mice, it has been proposed that E-selectin ligands and α4 integrin cooperate in HSC adhesion to perivascular niches.

### Proliferation versus quiescence

**Endosteal niches promote HSC quiescence**

The balance between HSC proliferation and quiescence is likewise regulated by several pathways. In the endosteal niche several interactions, involved in the maintenance of HSC quiescence, have been identified: Tie2/angiopoietin-1 (Ang-1); thrombopoietin (THPO)/MPL; Opn/OpnR; parathyroid hormone (PTH)/PTH receptor (PTHr) and Notch1/Jagged1. This pathway is also involved in promoting HSCs proliferation in the perivascular niche.

### Perivascular niches promote hematopoietic stem cell proliferation and self-renewal

In the vascular niche, HSC proliferation is associated with THPO/c-mpl and Wnt/β catenin pathway. THPO is expressed on BM stromal cells and acts synergistically with erythropoietin to promote erythroid progenitors and megakaryocytes proliferation. THPO stimulates c-myc mRNA expression through a PI3K- and MAPK-dependent pathway, thereby promoting HSC proliferation. Wnt proteins are expressed by BM stromal cell and exposure to Wnt was shown to stimulate proliferation and self-renewal of HSCs in vitro.

#### Differentiation

**Perivascular niches mediate hematopoietic stem cell differentiation**

Differenciation of HSCs occurs only in the perivascular niches and is mediated by FGF-4; SDF-1; VCAM-1/coc ; VE-cadherin and Notch1 pathway. SDF-1 is necessary for myelopoiesis and B lymphopoiesis, as shown by the severe reduction of B lymphopoiesis and lack of BM myelopoiesis in CCR4- and SDF-1 deficient mice. SDF-1 and FGF-4 promote megakaryocyte maturation and platelet production: FGF-4 supports the adhesion of megakaryocytes to sinusoidal BM endothelial cells (BMECs), thereby enhancing their survival and maturation, while SDF-1 augments platelet production by promoting their migration across BMECs. VE-cadherin is essential for VCAM-1 expression in BMECs, which in turn is required for FGF-4 mediated adhesion and SDF-1-induced transendothelial migration of megakaryocytes. Neutralizing antibodies to VE-cadherin decrease the localization of megakaryocytes to the vascular niche and disrupt megakaryocyte maturation and thrombopoiesis.

**Table 1. HSC properties are regulated by molecular cues conveyed by the bone marrow endosteal and vascular niches.**

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### References

- Pagepress, [Hematology Reviews 2009; 1:e14]{#page81}
Hemopathies require the support of aberrant bone marrow niches

Several hemopathies are characterized by a pre-malignant phase that progresses to a malignant phase. The molecular basis of this progression remains poorly understood. The data in the literature suggest the likelihood of such progression is very low and a malignant clone can remain “stable” for years. Moreover, in several diseases both phases are characterized by virtually the same genetic changes. Taking into consideration these aspects, it is legitimate to speculate that the genetic changes are necessary for the immortalization of a malignant clone but insufficient to promote the progression to a malignant phase. So other factors must take part in the progression.

The role of the hematopoietic BM microenvironment in malignant progression has been studied extensively and its importance was well illustrated in recent, in vivo, studies. Widespread inactivation of retinoblastoma protein (Rb) resulted in myeloproliferative disease, characterized by extramedullary hematopoiesis and increased mobilization and differentiation of HSCs from the BM. The phenotype was not recapitulated upon inactivation of Rb in HSCs maintained in wild-type environment. Moreover, Mx-Cre+Pten+/−/Cnice develop rapid and aggressive myeloproliferation that progressed to leukemia in 4-5 weeks post deletion. When Pten deletion was active in the context of a wild-type BM microenvironment, phenotypic and functional HSCs were lost without evidence of myeloproliferation or transformation. Finally, BM from wild-type mice transplanted into mice with a deficient retinoic acid receptor γ (RARγ) microenvironment rapidly develop myeloproliferative syndromes (MPS). These results strongly support the notion that the progression of the hemopathies is not entirely cell autonomous but depends on interactions between malignant cells and the BM microenvironment (BMM). As described above, BM niches support HSC properties such as adhesion, quiescence, chemotaxis and differentiation, and regulate the balance between self-renewal and differentiation. The idea outlined in this review is that alteration of the two BM niches, triggered by the aberrant expression of key molecules or cellular cues between the endosteal and the perivascular niche, impairs HSC responses, contributing to the progression of hemopathies. In chronic myeloid leukemia (CML), myelodysplastic syndromes (MDS) and multiple myeloma (MM) circulating endothelial cells (CECs), mobilized from the BM, share chromosomal aberrations with the malignant hematopoietic cells. These malignant CECs suggest the presence of aberrant niches in the BMM. Moreover, in B-cell lymphomas, identical genetic aberration could be found both in malignant cells and in the microvascular BM endothelial cells.

Adhesion

Ablation of HSC mobilization resulted in impaired drug resistance (CAM-DR)

It has been demonstrated, in vitro and in vivo, that cell-cell adhesion between hematopoietic cells and components of the BM niches, such as stromal cells, is involved in drug resistance in AML. ABL resistance to chemotherapy seems to be promoted by the adhesion-dependent secretion of WNT antagonists by osteoblasts. CAM-DR is mediated by integrins αv and β1, as shown in MM, CML and AML cell lines. Direct correlation has been found between the expression of integrins that mediate adhesion to FN and drug resistance. Coculture of ALL cell lines with BM stroma cells induced a reduced apoptosis by etoposide in this stroma model, drug resistance required direct cell-cell contact, since it could not be conferred by the addition of stromal conditioned media. Moreover, the presence of BMSCs during treatment of myeloma cell lines significantly decreases the apoptosis during exposition of mitoxantrone, an inhibitor of topoisomerase II. Notch-1 signaling seems to be involved in protection of MM from drug-induced apoptosis: overexpression of Notch-1 in Notch-1(−) myeloma cells up-regulated p21 and resulted in protection from drug-induced apoptosis. BM niches may provide a survival advantage for malignant cells following initial drug exposure and facilitate the acquisition of acquired drug resistance, determining disease relapse following chemotherapy.

Ablation of BM niches show impaired adhesive capacity, leading to a loss of quiescence and consequently to expansion of malignant hematopoietic stem cells

It has been hypothesized that HSC mobilization results from impaired adhesion to BM niches, allowing their migration into the peripheral blood, spleen and other extramedullary sites. This could explain the increase in circulating CD34+ cells reported in primary myelofibrosis (PMF) patients. The impaired adhesion could be explained by several mechanisms.

Altered expression of membrane adhesion molecules and integrins. For example, HSCs of CML patients have reduced adhesion molecules expression including L-selectin, CD44 and N-cadherin. This decrease correlates with, in vitro, reduced adhesive capacity of HSCs from CML patients. A disruption of CXCR4/SDF-1 axis. In idiopathic myelofibrosis (IM) the constitutive mobilization of CD34+ cells could be the conse-
quence of the creation of a proteolytic microenvironment within the BMM. It has been shown that malignant cells and the BMM produce metalloproteinase. Thus, the increased production of metalloproteinase-9 might disrupt adhesive interaction between CD34+ HSCs and BM niches through degradation of SDF-1 or cleavage of its receptor CXCR4, leading to the release of the HSCs into the peripheral blood.

**Proliferation vs. quiescence**

Aberrant niches determine an imbalance between proliferation and quiescence, accelerating the onset and progression of malignancy.

BM cells display a different set of adhesion molecules, extracellular matrix elements, growth factors and chemokines. Spleen fibroblasts isolated from PMF patients, in contrast to primary fibroblasts purified from the spleen of healthy subjects, are able to support the proliferation of autologous patient CD34+ cells, but not that of their normal counterparts. Moreover, it has been shown that somatic mutations that occur in BM stromal cells, such as p53 mutations, render these cells supportive of ALL growth. Finally, aberrant vascular niches produce several factors, such as VEGF, IL-6; granulocyte-macrophage and granulocyte colony-stimulating factors, that are able to support malignant hematopoiesis.

For example, it has been shown that coculture of AML cells with microvascular endothelial cells increases proliferation and inhibits apoptosis of AML cells.

Providing self-renewing and proliferative cues to malignant HSCs. ALL stromal cells regulate self-renewal and proliferation of a Philadelphia-chromosome positive (Ph+)VE-cadherin+ subpopulation of leukemia cells by promoting the expression of VE-cadherin, stabilizing β-catenin and up-regulating BCR-ABL transcripts. This way, due to the stromal support, malignant cells circumvent the requirement of exogenous Wnt signaling for self-renewal. Human MM cells also become independent of the IL-6/gp130/STAT3 survival pathway when cocultured in the presence of BMSCs. This evidence confirms the idea that BMSCs can provide alternative survival and proliferative signals to BM malignant cells.

**Angiogenesis**

The branching of new microvessels from pre-existent blood vessels, is kept at set point in which there is a balance between pro- and anti-angiogenic molecules. The angiogenic switch, unbalanced set point in favor of pro-angiogenic molecules, favors the production of new microvessels.

Increased angiogenesis has been described in a number of hematopoesis. The extent of BM neo-vascular formation correlates also with patient prognosis and these hemopathies are sensitive to anti-VEGF and VEGF receptor treatments. The expanded BM endothelium may support malignant HSC growth by protecting them from chemotherapy-induced apoptosis and/or promoting their proliferation in a paracrine way through the release of factors such as G-CSF, IL-10, IL-6 and vascular endothelial growth factor C (VEGF-C).

Aberrant vascular niches can induce quiescence in malignant cells playing a role in tumor maintenance.

Adhesion of malignant HSCs to BMSCs may induce quiescence by inhibiting cell proliferation. For example, Notch-1 activation in MM cells, after incubation on BMSCs, results in the accumulation of the cells in G0/G1 phase of cell cycle. Aberrant niches may thus contribute to the maintenance of a malignant pool of HSCs.

**Differentiation**

Aberrant niches can induce malignant transformation of normal hematopoietic stem cells.

The donor cell leukemia (DCL), a hematopoietic cell, is apparently the result of malignant transformation of normal donor hematopoietic cells in the transplant recipient. One of the hypotheses is that the host microenvironment in which the original malignancy developed may trigger malignant transformation in donor cells, favored by the immunocompromised status after transplantation and by perturbation of the host BM following multiple rounds of chemotherapy.

**Conclusions**

This paper highlights the key data demonstrating that changes in the signals delivered by BM endostele and/or perivascular niches may lead to an impairment of survival, differentiation and proliferation of HSCs. Thus, aberrant BM niches participating in HSC regulation contribute in a crucial way to the progression of hemopathies. Therefore, the molecular cues that contribute towards BM niche alteration during the onset and development of hemopathies represent a new challenging therapeutic target.

[Hematology Reviews 2009; 1:e14]
References


31. Petit I, Spyker-Kravitz M, Nagler A, et al. G-CSF induces stem cell mobilization by...
66. Andreasson B, Swolin B, Kutti J. Patients with idiopathic myelofibrosis show increased CD34+ cell concentrations in peripheral blood compared to patients with...
85. Bruns I, Cizbure A, Fischer JC, et al. The hematopoietic stem cell in chronic phase CML is characterized by a transcriptional profile resembling normal myeloid progenitor cells and reflecting loss of quiescence. Leukemia 2009; Jan 22 [Epub Ahead of Print].
A sensitivity comparison of the Quick and Owren prothrombin time methods in oral anticoagulant therapy

Juha Horsti
Department of Clinical Chemistry, Centre for Laboratory Medicine, Tampere University Hospital, Tampere, Finland

Abstract

Prothrombin time (PT) is the leading test for monitoring oral anticoagulation therapy (OAT). According to the World Health Organization recommendation, International Normalized Ratio (INR) results obtained from the same patient samples with the major PT methods (Quick and Owren) should be the same when the therapeutic range is the same. In our study blood samples were obtained from 207 OAT patients. We analyzed the samples using two Quick and two Owren PT (combined thromboplastin) reagents for INR and assessed the sensitivity and true coagulation activity using a new-generation PT method. The INR values with the Quick PT and Owren PT methods were very similar around the normal range, while unacceptable differences were seen within the therapeutic range and at higher INR values. The Quick PT results as INR are clearly lower than those given by Owren PT and the difference increases toward higher INR. The new PT method functions well with both Owren PT reagents, and we can calculate the true active INR. The Quick PT methods show no sensitivity to coagulation inhibition measurement. The harmonization of the INR is an important goal for the safety of OAT patients. More accurate INR results reduce morbidity and mortality, and the therapeutic ranges should be similar worldwide. In this study we found unacceptable differences in INR results produced by the two PT methods. The new method showed a lack of sensitivity to Quick PT. For the global harmonization of OAT therapy and for INR accuracy only the more sensitive Owren PT method should be used.

Introduction

Oral anticoagulation therapy (OAT) is one of the most commonly used medications worldwide. The purpose of the treatment is to balance the risks of hemorrhage and thrombosis in the patient. Arterial and venous complications commonly are involved in morbidity and mortality in OAT patients globally. The vitamin K antagonists coumarin and warfarin are inexpensive and the most widely used medicines in the prevention and treatment of thromboembolism in various clinical situations, and the benefit of OAT has been proved. The major drawback with warfarin is a narrow therapeutic window and individually variable responses to the treatment. Thus frequent prothrombin time (PT) checks are required to ensure that anticoagulation remains within the therapeutic range, which is 2.0-3.0 International Normalized Ratio (INR).

New medications for oral anticoagulation therapy have been developed over a number of years and anticipated without laboratory test control in an effort to replace warfarin. The new medicines, however, have not proved superior to warfarin. The new molecules are expensive and involve serious side effects or only narrow indications for OAT. According to a recent review, prothrombin time has served as a basis for OAT monitoring since its first description by Quick more than 70 years ago. PT measures vitamin K-dependent coagulation factors II, VII, and X. Warfarin (VKAs) inhibits the synthesis of coagulation factors II, VII, IX, and X in the liver, which remain partially inactive unless nine to 13 of the amino-terminal glutamate (Glu) residues are carboxylated to form Ca2+-binding γ-carboxyglutamate (Gla) residues. Thus it is inevitable that the more medication is administered, the more inactive coagulation factors will result, hampering and inhibiting the PT measurement.

Today, two major PT methods are accepted for anticoagulant medication control and they are used globally for chronic anticoagulation checks: either the Quick PT, which is based on the technique described by Quick and his colleagues in 1935, or the Owren PT (combined thromboplastin reagent). The Owren PT is the predominant approach used in the Nordic countries, the Benelux, and Japan. The accuracy and comparability of PT results are essential to the safety of the individual in anticoagulant therapy and to improve the applicability of anticoagulation guidelines. The World Health Organization (WHO) recommendation for the use of INR given in the mid-1980s was aimed to harmonize PT results for OAT, regardless of the laboratory, reagent, instrument, or method used.

Unfortunately, an increasing body of evidence indicates that this goal has not been achieved. Different reagents are known to vary with respect to the source of thromboplastins and other reagent components, and these have not been characterized in detail. The Owren method is regarded as the “reference method” in which fibrinogen and factor V are added to the reagent, and these do not depend on K-vitamin antagonists. The Quick PT measures both of these coagulation factors, which is a drawback in OAT control. In the Owren method the sample volume in the reaction mixture is 5% only, in the Quick method 33%. On account of this difference in sample dilution the Owren PT is considered to be a more sensitive method than the Quick PT.

In the present study, we sought to compare INR results for warfarin therapy from the same OAT patient samples using both the Owren and the Quick PT methods. The aim was to study the method and reagent sensitivity of Owren and Quick PT using the new-generation PT method developed by Horsti et al., which can measure active coagulation without inhibition from inactive coagulation factors.

Materials and Methods

Patients and blood sampling

Venous blood samples were obtained from 207 hospital and health-center patients referred to the PT test for the monitoring of oral anticoagulant therapy. In our region a “P-INR” test code is used for this purpose. Hence, the patient samples represented all possible phases of anticoagulation: pretreatment, dose-adjusting phase, and steady-state phase. All the procedures were approved by the responsible committee of our institution in accordance with the Helsinki Declaration of 1975. The blood (3.15 mL) was drawn into citrate coagulation tubes (Greiner Labortecnik GmbH, Vacuette cat. No. 454332, 9NC) containing 0.35 mL 0.109 mol/L (3.2%) citrate solution. The sample needle (Terumo, Venoject needle, Quick Fit, cat. No. MN-2138MQ) was 0.8x40
mm. The sample tubes were centrifuged at 1850 g for 10 min at 20°C to separate the plasma. All the measurements were commenced within eight hours from blood collection.

**Prothrombin time determination**

The calculation formula is for the International Normalized Ratio: INR = (sample – normal –)/normal, where ISI is the International Sensitivity Index. When ISI is close to 1.0, the reagent is sensitive and ISI is insignificant in the INR calculation. The PT coagulation times were measured using a fully automated ACL TOP CTS coagulation analyzer (Instrumentation Laboratory, IL, Lexington, MA, USA). For the Owren PT (combined thromboplastin reagent) the coagulation reaction contained 10 µL of citrated sample plasma, 60 µL of diluent, and 140 µL of reagent. The volumes for “dilution measurement” were 5 µL + 65 µL + 140 µL. The test reagents were Nycotest PT, cat. No. 1002488 (rabbit brain thromboplastin) and diluent (Nycotest PT, dilution liquid, cat. No. 999002) from Axis-Shield as lot. 10132661, ISI=1.12; Owren’s PT, cat.no. GHI 131-10 (rabbit thromboplastin), and diluent (Owen’s buffer, cat.No. GHI 150) from Medirox as lot 75051, ISI=1.16. For the one-stage prothrombin time with Quick, 100 µL of coagulation reagent was added to 50 µL of citrated plasma. Sample volumes for the dilution were 100 µL + 25 µL + 25 µL (Factor Diluent, HemoSII, cat. No. 0009576900 from IL). The test reagents were: RecombiPlasTin 2 G cat. No. 0020003050 (recombinant human tissue thromboplastin) from Instrumentation Laboratory, Lexington USA as lot. N0486316, ISI=0.97; PT-Fibrinogen HS PLUS cat. no. 0008462801 (rabbit brain thromboplastin) from Instrumentation Laboratory, Lexington USA as lot. N0185573 ISI=1.11.

**Additional reagents**

Control Plasma Normal cat.No. 1002387 from Axis-Shield as lot. 1D71AOA, NKP Normal Control Plasma cat.No. GHI 162 from MediRox AB as lot. 72011, and Normal Control Assayed cat.No. 0020003110 from IL as lot. N0386069.

**The new-generation prothrombin time method**

We constructed PT (sec) vs. C (C = plasma or calibrator dilution factor) plots for normal and OAT plasmas. This is consistent with an uncompetitive inhibition principle with oral anticoagulants. From the line equation the -axis intercept is calculated. This is the so-called minimal clotting time (tICC) with an infinite number of clotting factors. The difference in intercepts (y-axis) between normal plasma and OAT plasma indicates the action of uncompetitive inhibition in seconds without a calibration effect. We went on to calculate the difference in intercepts also in INR units and subtracted this from total INRtot (more explanation about the new method in reference 28 [patent pending for PT method]).

### Table 1. Differences in INR results between four PT methods at variable INR levels as INR units and percentages. The correlation equations between methods were used for the calculations.

<table>
<thead>
<tr>
<th>Method</th>
<th>INR</th>
<th>Diff %</th>
<th>Diff %</th>
<th>Diff %</th>
<th>Diff %</th>
<th>Diff %</th>
<th>Diff %</th>
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</thead>
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<tr>
<td>Nycotest PT</td>
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<td>1.00</td>
<td>0.05</td>
<td>0.00</td>
<td>1.08</td>
<td>8.03</td>
<td>-</td>
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<td>Owren’s PT</td>
<td>2.00</td>
<td>1.90</td>
<td>5.25</td>
<td>0.11</td>
<td>1.88</td>
<td>6.23</td>
<td>0.12</td>
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<tr>
<td>PT-Fib HS</td>
<td>3.00</td>
<td>2.79</td>
<td>6.98</td>
<td>0.21</td>
<td>2.67</td>
<td>10.99</td>
<td>0.33</td>
</tr>
<tr>
<td>Recomb 2G</td>
<td>4.00</td>
<td>3.69</td>
<td>7.85</td>
<td>0.31</td>
<td>3.47</td>
<td>13.37</td>
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<td>Analyse-it</td>
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<td>Microsoft Excel</td>
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<td>5.48</td>
<td>8.72</td>
<td>0.52</td>
<td>5.06</td>
<td>15.75</td>
<td>0.94</td>
</tr>
</tbody>
</table>

**Results**

We compared the results of four different commercial INR determination methods (two Quick PT and two Owren PT, ISI values 0.97; 1.11 and 1.16) from 207 blood samples from patients in imminent or ongoing OAT. All the ISI values used for reagents were manufacturer values. The INR values obtained with the different methods were similar around the normal range or INR 1 (Figure 1). In contrast, marked differences were seen in the therapeutic range (2.3 INR) and higher INR values between the Quick PT and Owren PT methods. The INRs produced by the Quick PT are clearly lower than those given by the Owren PT, and the difference increases toward higher INRs. Using correlation equations between the four methods, the differences were calculated applying the Microsoft Excel 5.0 and Passing & Bablok method comparison, Analyse-it for Microsoft Excel from Analyse-it Software Ltd programs (Table 1).

The difference between the Owren PT methods in INR terms is below 10% over the whole measuring range. In the therapeutic range, the average difference is 0.16 INR; 6.10%. The difference between Nycotest PT and Pt-Fib HS is 8.03-15.75% and in the therapeutic range an average of 0.23 INR; 8.6%. The difference between Nycotest PT and Recomb 2G is 0.74-27.43% and in the therapeutic range an average of 0.49 INR; 19.0%.

The Owren methods showed excellent correlation (y = 0.96 x + 0.052; intercept 95% CL 0.034 to 0.071; slope 0.948 to 0.973), while the correlations between Quick and Owren PT were not good: Nyco PT vs. Pt-Fib HS (y = 0.7955 x + 0.2802; intercept 95% CL 0.242 to 0.319; slope 0.769 to 0.823), and Nyco PT vs. Rec 2G (y = 0.669 x + 0.3357; intercept 95% CL 0.290 to 0.381; slope 0.642 to 0.700). The correlation was not good either between the Quick methods: Pt-Fib HS vs. Rec 2G (y = 0.8267 x + 0.1055; non-linear relationship; intercept 95% CL 0.063 to 0.150; slope 0.800 to 0.857).

The new-generation PT method functions well with the two Owren PT methods and makes it possible to calculate the true active INR without inhibition (Figure 2). The correction lowers the INR values closer to Quick PT values. Marked individual differences were seen in the therapeutic range 2.3 INR and higher values between Rec 2G INR (Quick PT) and Owren’s PT INRACT (Owren PT) (Figure 3). The correlation between active INR results is good (y = 0.895 x + 0.13; intercept 95% CL 0.078 to 0.122; slope 0.885 to 0.907). The Quick PT methods show no sensitivity to the coagulation inhibition measurement.

**Discussion**

The global harmonization of INR and therapeutic ranges for different clinical indications is an important goal for the benefit of patients...
Figure 1. INR values in increasing order from 207 analyzed patient samples on anticoagulation therapy. Nycotest PT and Owren’s PT are Owren methods (combined thromboplastin reagent), and PT-FibHS and Rec 2G are Quick methods.

Figure 2. INR_{TOT} and INR_{ACT} values in increasing order from 207 analyzed patient samples on anticoagulation therapy. Owren’s PT reagent (combined thromboplastin reagent) and the new-generation PT method are used for INR measurement.

Figure 3. Rec 2G, Quick PT, INR, and Owren’s PT (combined thromboplastin reagent) INR_{ACT} values in increasing order from 207 analyzed patient samples on anticoagulation therapy. The new-generation PT method is used for INR_{ACT} measurement.
as well as clinicians. WHO originally envisaged INR as a reliable and safe method for obtaining results from anticoagulation trials and as a basis for expert guidelines for treatment. However, the results of earlier studies and this present study demonstrate that the goal has not been achieved. The narrow therapeutic range and the increase in complications and mortality outside this range call for the development of more accurate laboratory analytics. A computer simulation study of serial INR measurements has been conducted within the most widely used therapeutic range (INR 2.0 - 3.0); the authors concluded that analytical imprecision should be <5% and analytical bias < ± 0.2 INR units.29

In this study, agreement between the Quick PT and Owren PT methods was particularly poor and failed to meet the qualification requirements. For this study we selected a newly developed recombinant PT reagent, but the result was not acceptable. We may wonder whether the difference in sample volume in the reaction mixture (5% and 33%) accounts for the measurement sensitivity. Both Owren PT reagents are sensitive to inactive coagulation factor measurement (inhibition), while the Quick PT reagents are not.

In our earlier study we used DadeBehring Innovin with human placenta thromboplastin (Quick PT reagent), which is the most sensitive reagent to inactive coagulation factors and inhibition. The sensitivity thus does not depend only on differences in principle between the Quick PT or Owren PT methods. The thromboplastin and other components used in the reagent affect the reagent sensitivity. Horsti24 has compared the Quick PT and Owren PT methods for the harmonization of INR results and concluded that Quick PT yields clinically divergent and Owren PT clinically acceptable INR results. It would be interesting to study the effect of lupus anticoagulants on Quick PT and Owren PT methods. The results of recent studies, unfortunately, demonstrate that attempts at harmonization have to improve.22 The unacceptable situation for oral anticoagulation was confirmed further by this present investigation.

How can the harmonization of INR results be improved worldwide? For global use, WHO should recommend only the Owren PT, which is the superior method. The advantages of Owren PT are the fact that it measures only vitamin K-dependent coagulation factors F II, F VII, and F X (not fibrinogen and F V), and provides greater sensitivity and extensive dilution of the interfering matrix substances in the final reaction mixture.18,29 The patient samples also contain inactive coagulation factors, the amount of which increases concomitant with increased anticoagulant medication and higher INR levels, interfering with and causing error in the coagulation measurement. The inhibition depends on the medication and the patient’s individual metabolism, and thus each patient’s sample must be corrected individually.25,26 The Owren PT methods are sensitive, but more susceptible to the inactive coagulation factors and inhibition in measurement. In an earlier study we measured only the active coagulation factors, INRact (F II, F VII, FX) without inhibition, which provided a new possibility to develop anticoagulant therapy and more appropriate care for OAT patients. The active coagulation factors are responsible for thrombosis or bleeding in vivo and medication should be based on this principle.22,24 The inhibition interferes with the calibration procedure and measurement.

This study was conducted from the point of view of a clinical laboratory without clinical data and outcomes are missing between methods (Quick PT, Owren PTTov, Owren PTov), which would give the final answer as to the superiority of PT methods. This study revealed the lack of sensitivity in both Quick PT methods but good sensitivity and correlation in the Owren PT activity (INRAct) measurements. The editors of Clinical Chemistry have posed the critical question and sought answers: “Has the Time Arrived to Replace the Quick Prothrombin Time Test for Monitoring Oral Anticoagulant Therapy?” On the basis of recent studies and our opinion we can answer: “Yes, with the Owren PT without inhibition.”

References

23. Horsti J. Agreement of owren and quick prothrombin times: effects of citrate and
Clear cell renal cell carcinoma with vaginal and brain metastases: a case report and literature review

Tobe Momah,1 Etwaru Dhanan,2 Phillip Xiao,1 Vasantha Kondamudi1
1PGY-II Department of Family Medicine; 2Department of Urology; 3Department of Pathology; Brooklyn Hospital Center, Brooklyn, New York, NY, USA

Abstract

There are very few cases of clear cell renal cell carcinoma with metastases to the vagina and brain reported in the literature. Our case study highlights this rare clinical occurrence and its associated complications including pulmonary embolism. In addition we discuss current management guidelines for treating and diagnosing the disease, and how this management improves prognosis.

Case report

Presentation and history
A 55-year-old African American female with a past medical history of hypertension, nicotine dependence (stopped smoking eleven years ago), and Para 2022 (status post-right salpingectomy for ectopic pregnancy) presented with six weeks of persistent postmenopausal bleeding per vagina (PV). Prior to presentation the patient had been menopausal for ten years and used only amiodipine and avaldide tablets as medications. She has a positive family history (sister) of breast cancer and denied intra-partum exposure to diethyl stilbestrol (DES). The physical examination revealed a loose necrotic mass with a pedunculated polyp attached to the posterior wall of the vagina and measuring 3.5x1.5x1 cm. Endocervical and endometrial curettings were negative for malignancy while polypectomy of the vaginal mass revealed foci of necrosis, surface ulceration, and a polypoid tumor consistent with clear cell carcinoma of the vagina (Figure 1).

Initial management
A computerized tomography (CT) scan of the abdomen with contrast had been done four months previously and showed a 4.4 cm left kidney mass with peripheral enhancement. A CT scan of the chest, abdomen, and pelvis done after the diagnosis of clear cell malignancy revealed a pulmonary embolism (PE) in the left lower lobe pulmonary arteries, an enlarged area of enhancement measuring 7 cm in the inferior aspect of the left kidney (Figure 2), and a 6x5.6 cm focus of soft tissue enhancement in the region of the vagina (Figure 3). These findings were suggestive of cystic renal cell carcinoma and metastatic clear cell carcinoma of the vagina, respectively.

Treatment
The patient was started on Lovenox (enoxaparin) and coumadin, but she started bleeding PV a week later and was admitted to our inpatient service with shortness of breath, and for anticoagulation treatment and inferior vena cava filter placement. She later underwent a laparoscopic hand-assisted left nephrectomy with histopathological examination of the 6.6x6x4.5 cm left lower kidney pole specimen, which was consistent with renal clear cell carcinoma (Figure 4). The tumor extended into the left perirenal adipose tissue, was a Fuhrman nuclear grade G3 tumor, involved the left renal vein, and was staged as T3N0Mx secondary to the vaginal metastases.

Clinical course and outcome
The patient tolerated surgery well, and after discharge received palliative radiation therapy (4860 cGy in 27 fractions of 180 cGy) to the pelvis. However, she was re-admitted three months after discharge for persistent headaches and right-sided upper extremity numbness and weakness, which on CT of the head was found to be because of a secondary post-pelvic parietal mass. She underwent an intracranial incision biopsy of the left parietal brain tumor that showed metastatic clear cell carcinoma histologically, consistent with a renal primary tumor. She received postoperative therapeutic irradiation of the cranium for metastatic brain lesions and recently was started on chemotherapy (Sutent 50 mg once daily orally). She continues taking her antihypertensive medications but had to discontinue her anticoagulation drugs because of recurrent PV bleeding. She currently has good performance status and otherwise is stable medically.

Discussion

Epidemiology
Clear cell renal cell carcinoma (RCC) is the most common primary site for clear cell tumors, yet clear cell RCC metastases to the vagina or brain are rare with only one case of the former reported in the literature to date.1 Eighty cases of RCC with vaginal metastases have been reported in the literature,2 and because clear cell carcinomas constitute about 80% of all renal cell carcinomas,2 most of these cases may be clear cell carcinomas in fact, but are under-reported as such. Clear cell RCC usually occurs in the sixth or seventh decade of life and is three times more common in men than in women.1

Clinical presentation
The most common symptom on presentation in RCC cases with vaginal metastases is vaginal bleeding,1,3 and its most common sites for metastases include extra-renal regions like lung, lymph nodes, liver, and bones.1 Our case was unique for its metastatic sites, clinical presentation, and associated complications. Clear cell RCC with metastases to the vagina and brain is very uncommon with one case of the former1 and none of the latter reported in the literature. In postmenopausal women only one case of primary clear cell carcinoma of the vagina has been reported4 because these tumors tend to occur more in young patients exposed to DES in utero.4 Therefore, a solitary lesion of clear cell carcinoma in the vagina in a postmenopausal patient is more likely to be a metastatic secondary than a primary lesion, and a consideration of clear cell tumor primary sites should be performed immediately. This was done in our case and revealed a primary site in the left kidney, which is typically the primary site for clear cell RCC as the retrograde filling of the left renal vein from the left ovarian vein and uterovaginal plexus eventually involves the left kidney.2

Complications
The vascular effects of clear cell RCC include the intravascular growth of the tumor into the left renal vein, and may have caused the tumor embol to the left lower lung pulmonary arteries that resulted in our patient’s pulmonary embolism. The vascular involvements of clear cell carcinoma are well documented in the literature5 but this is the first reported case with an associated pulmonary embolism.
Diagnostic methods
Fine needle aspiration cytology to establish the anatomic site of origin in metastatic clear cell RCC cases, when combined with clinical features, is 95% accurate and is easier to administer in outpatient settings and follow-up cases than are endometrial biopsies. However, the use of magnetic resonance imaging (MRI) and CT scans still provide the mainstay of primary clear cell RCC diagnoses (and subsequent diagnoses of secondary metastases) owing to their greater utility and accuracy.

Treatment
The use of whole body radiation therapy in cases involving a solitary metastatic site clear cell carcinoma currently is not recommended as adjuvant loco-regional radiation therapy is the preferred treatment for clear cell RCC. In diffuse or non-resectable disease, or when paraneoplastic syndrome, severe hemorrhage, or severe pain is present, however, the use of palliative nephrectomy is recommended.

Prognosis
Multiple metastases, recurrent metastases, and renal vein involvements are poor prognostic indicators for survival and, as seen in our patient, are prone to develop complications like pulmonary embolism. Patients with multifocal clear cell RCC (like our patient with two metastatic sites) are more likely to have a contralateral recurrence than are patients with solitary clear cell RCC (risk ratio: 2.91, p=0.142).

Conclusions
The development of recurrent shortness of breadth in a clear cell RCC patient with vaginal metastases is indicative of pulmonary embolism, and the presence of neurological deficits is suggestive of brain metastases. A high indicative index for a clear cell primary tumor is a requirement for the diagnosis of a secondary clear cell vaginal malignancy and for avoiding complications like pulmonary embolism, further metastatic spread, and the consequent deterioration of the patient.

References
A case of follicular lymphoma complicated with mesenteric panniculitis

Yotaro Tamai,1 Osamu Imataki,2 Ichiro Ito,3 Kimihiro Kawakami1

1Division of Hematology & Stem Cell Transplantation, Shizuoka Cancer Center, Shizuoka, Japan; 2 Division of Hematology, Department of Internal Medicine, Faculty of Medicine, Kagawa University, Kagawa, Japan; 3Division of Pathology, Shizuoka Cancer Center, Shizuoka, Japan

Abstract

Mesenteric panniculitis (MP) is a rare disease occasionally complicated with lymphoma. A 55-year-old female presented with MP accompanied by malignant lymphoma. This patient was first treated for follicular lymphoma and subsequently for panniculitis. After 6 courses of R-CHOP chemotherapy, the treatment response was partial. An additional course of salvage chemotherapy led to a complete response. Since the mesenteric mass progressed simultaneously with the regression of other lymphoma lesions, we performed a biopsy of the mesenteric mass and pathologically confirmed an MP lesion without lymphoma. Subsequent high-dose chemotherapy led to CR and the MP lesion remained stable. In the present case, MP progressed with chemotherapy. We concluded that mesenteric lesions suspected of progressing or recurring should be diagnosed pathologically even if asymptomatic.

Introduction

An extensive involvement of mesenteric fat tissue of the small bowel with chronic non-specific inflammatory disease, first reported by Jura et al. in 19241 was described, detailed, and named as mesenteric panniculitis (MP) by Ogden et al. in 1960.2 MP is always discussed with pathological findings concerning its etiology and 3 components of histology, fibrosis, chronic inflammation, and fat necrosis, suggesting various mechanisms of disease (called sclerotic mesenteritis, mesenteric panniculitis, and mesenteric lipodystrophy, respectively).2 Histologically, MP lesion is characterized by the infiltration of mesenteric fat tissue by inflammatory cells, mainly consisting of lymphocytes and plasmacytes, along with a mixture of fat necrosis and fibrosis.3 Although the diagnosis of MP before biopsy is primarily based on guesswork, computed tomography (CT) findings are helpful for cases in which mesenteric biopsy is problematic.4 The clinical course of MP is usually benign, and spontaneous remission is quite common without specific treatment.5

The pathogenesis of MP remains obscure and many associated diseases are known, including mechanical stimulation such as surgery or trauma, infections, vascular diseases, and malignant and non-malignant neoplasms.1 The occurrence rate of lymphoma as an underlying disease is 15% or more, and no malignant involvement of the mesentery was diagnosed in almost all cases.1 In a case series of MP accompanied with malignant lymphoma, Kipfer et al. (8 cases) suggested that mesenteritis was a non-specific response to underlying lymphoma.5 Other reports introduced MP incidence during the clinical follow-up course.
of lymphoma. Ogden (2 cases) and Perez-Ferriols (1 case) both reported on the association of these 2 diseases.

Case Report

A 55-year old woman presented with right cervical lymph node swelling in July, 2004, followed by inguinal lymph node swelling a few weeks later. She was admitted to our hospital in August, 2004. The results of the blood and bone marrow examinations performed at that time are shown in Table 1. A lymph node biopsy of the inguinal area revealed grade 2 follicular lymphoma. CT images of her neck, thorax and abdomen, and FDG-PET revealed stage IV disease. She was diagnosed as having stage IV lymphoma with femoral bone involvement, as confirmed by femoral MRI. Her follicular lymphoma international prognostic index (FLIPI) was intermediate (stage and lymph-node lesions). IgH-BCL rearrangement (FISH) was detected in her lymph nodes and bone marrow cells, although neither morphological abnormality nor apparent involvement of lymphoma cells were revealed. We did not examine her for IgH-BCL rearrangement of peripheral blood. B symptoms were absent; however, since she complained of bone pain and it was progressive, we decided to treat her with chemotherapy. R-CHOP chemotherapy consisted of rituximab (375 mg/m², day 0), cyclophosphamide (750 mg/m², day 1), doxorubicin (50 mg/m², day 1), vincristine (1.4 mg/m², day 1), and prednisolone (100 mg/kg body weight, day 1-5), injected tri-weekly until 6 courses were completed. At this point only a partial response (PR) was observed through objective radiological evaluations (for nodal masses FDG-PET was negative and more than 50% regression was observed on CT). After R-CHOP 2 courses at a 3-week interval of the ACES regimen (cytarabine 2,000 mg/m² day 5, carboplatin 100 mg/m² day 1-4, etoposide 80 mg/m² days 1-4, methyprednisolone 500 mg/body days 1-5) were used as salvage chemotherapy, after which her disease was considered to be in CR (IgH-BCL rearrangement in her lymph nodes and bone marrow became negative). At the same time, a contrast-enhanced peritoneal mass appeared on CT imaging (Figure 1A) and FDG-PET (Figure 1B). We identified the pathological etiology of the peritoneal lesion proceeding to the peripheral blood stem cell transplantation (PBSCT) by CT-guided biopsy, which revealed mesenteric panniculitis (MP) not concomitant with the lymphoma lesion (Figure 1C). Her MP did not produce symptoms through her treatment course. However, the bone lesion recurred in June, 2005, when she received PBSCT. She has maintained CR with no progression of the MP mass for more than three years.

Discussion

Mesenteric panniculitis is a lipodystrophy characterized by non-specific inflammatory disease first reported by Ogden et al. Emory et al. reviewed 84 cases of MP pathologically and documented that a mixed histology of 3 components (fibrosis, chronic inflammation, and fat necrosis) existed in the lesions, which could be classified according to the dominant pathological changes, i.e. mesenteric lipodystrophy (ML) type, mesenteric panniculitis (MP) type, and sclerosing mesenteritis (SM) type. However, not all cases can be definitively categorized in these 3 subtypes because the histologically dominant subtype is often too varied to define as ML, MP, or SM, respectively. Due to this pathological variation and the various underlying diseases of MP, the etiology of this disease is thought to be a single pathology with various causes including immunological reactions, malignancies, infection, physical stimulation, or traumatic disorders.

Past retrospective analysis reported that, in general, MP occurs in patients in their 60s (median age of occurrence), has slightly higher incidence in men, is accompanied by abdominal pain, and is often associated with malignant lymphoma. Generally, isolated MP does not need to be treated. Kipfer et al. reported that 8 among 53 reviewed cases of MP with malignant lymphoma led to complications related to MP. They could find no pathological involvement of lymphoma in the panniculitis lesion in any case. In 3 of the 8 cases, MP existed with malignant lymphoma concomitantly. In 2 other patients, the lymphoma lesion was found two or three months later. Another 3 patients were diagnosed as having malignant lymphoma occurring in a different area from the MP lesion. These findings suggest that MP can precede or proceed to lymphoma. However, no panniculitis has been revealed in lymphoma lesions by biopsy, and no obvious association has been reported until now. In our case, the mesenteric lesion progressed and was detected by FDG-PET imaging during...

Table 1. Laboratory data on admission. The profile of the peripheral blood cell count was within the normal range. Biochemical data showed a normal LDH level and no organ disorders. Cytogram of bone marrow revealed no obvious morphological changes and the involvement of lymphoma cells was not detected.

<table>
<thead>
<tr>
<th>Component of blood cell</th>
<th>White blood cells</th>
<th>9100 /μL (3500–9100)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Granulocytes</td>
<td>69% (32–79)</td>
</tr>
<tr>
<td></td>
<td>Eosinophils</td>
<td>1% (0–6)</td>
</tr>
<tr>
<td></td>
<td>Basophils</td>
<td>2% (0–2)</td>
</tr>
<tr>
<td></td>
<td>Monocytes</td>
<td>3% (0–8)</td>
</tr>
<tr>
<td></td>
<td>Lymphocytes</td>
<td>25% (18–59)</td>
</tr>
<tr>
<td></td>
<td>Red blood cells</td>
<td>415x10^6 /μL (376–500)</td>
</tr>
<tr>
<td></td>
<td>Hemoglobin</td>
<td>12.6 g/dL (11.3–15.2)</td>
</tr>
<tr>
<td></td>
<td>Hematocrit</td>
<td>37.4% (33.4–44.9)</td>
</tr>
<tr>
<td></td>
<td>Platelets</td>
<td>23.1x10^12/μL (13.0–36.9)</td>
</tr>
</tbody>
</table>

**Biochemistry**

<table>
<thead>
<tr>
<th>Total protein</th>
<th>7.7 g/dL (6.7–8.3)</th>
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<tbody>
<tr>
<td>Albumin</td>
<td>4.6 g/dL (4.0–5.0)</td>
</tr>
<tr>
<td>Total bilirubin</td>
<td>0.4 mg/dL (0.3–1.2)</td>
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<tr>
<td>Glutamic pyruvic transaminase</td>
<td>19 IU/L (5–40)</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>105 IU/L (115–245)</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>259 IU/L (115–359)</td>
</tr>
<tr>
<td>Blood urea nitrogen</td>
<td>15.5 mg/dL (8.0–22.0)</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.6 mg/dL (0.47–0.79)</td>
</tr>
<tr>
<td>Urine acid</td>
<td>3.8 mg/dL (2.5–7.0)</td>
</tr>
<tr>
<td>Natrium</td>
<td>142 mEq/L (136–147)</td>
</tr>
<tr>
<td>Potassium</td>
<td>4.1 mEq/L (3.6–5.0)</td>
</tr>
<tr>
<td>Chloride</td>
<td>106 mEq/L (98–109)</td>
</tr>
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</table>

**Coagulation**

<table>
<thead>
<tr>
<th>Prothrombin time</th>
<th>97% (70–130)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated partial thromboplastin time</td>
<td>28.5 sec (24.3–36.0)</td>
</tr>
<tr>
<td>International normalized ratio</td>
<td>1.02 (0.85–1.15)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>274 mg/dL (150–400)</td>
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</table>

**Bone marrow**

<table>
<thead>
<tr>
<th>Number of nuclear cells</th>
<th>9.4 x10^10 /μL (10–20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Megakaryocytes</td>
<td>43.8 x10^10 /μL (50–150)</td>
</tr>
<tr>
<td>Myeloid/erythroid ratio</td>
<td>2.36 (2–4)</td>
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<tr>
<td>Blasts</td>
<td>0.2% (1.0–1.5)</td>
</tr>
<tr>
<td>Promyelocytes</td>
<td>0.6% (2.0–6.5)</td>
</tr>
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</table>
chemotherapy, which reduced to remission the lymphoma lesions (lymph nodes and femoral bone head). This discrepant course made it problematic for physicians to decide on a treatment plan. If we had regarded the mesenteric mass in our patient as refractory lymphoma, we would have had to administer a salvage regimen. If the mesenteric mass had been benign, we could have continued the existing regimen. A biopsy confirmed the remission of her lymphoma and resolved this problem. Autologous PBSCT brought about a durable CR in the patient. The MP lesion regressed after the planned treatments, with no recurrence for three years. Therefore, we recognized that her MP was chemotherapy-related panniculitis, since panniculitis is thought to be brought about by physical or chemical stimulation.5

Although FDG-PET was an effective and powerful tool for the staging, localization, and response assessment of malignant lymphoma, the differential diagnosis for lymphoma lesions with physiological accumulation or inflammatory change is sometimes difficult.10,11 Though FDG-PET/CT fusion imaging is more efficient for diagnosis of the active lymphoma lesion,10 a pathological diagnosis via biopsy is essential for cases in which a false positive is suspected. Zissin et al. reviewed 19 oncological patients with MP12 and reported that FDG-PET had high specificity (100%) among 11 FDG-PET-negative subjects and 88% sensitivity (7 out of 8 subjects). We concluded that mesenteric lesions suspected of progressing or recurring with positive uptake with FDG-PET should be diagnosed pathologically even if asymptomatic.

References
1. Jura V. Sulla mesenterite retrattile e sclerosante. Policlinico (sez. prat) 1924;31: 575-8
Massive pleural effusion due to IgG multiple myeloma

Kathryn J. Lang, Surjit Lidder, Robin Aitchison

1Department of Haematology, Wycombe General Hospital, UK; 2Department of Trauma and Orthopaedics, Royal London Hospital, Whitechapel, London, UK

Abstract

Pleural effusion directly attributable to multiple myeloma is exceedingly uncommon and is said to occur in only 1-2% of cases. Of these around 80% occur in IgA disease. We report a case of myelomatous pleural effusion (proven on cytological and immunohistochemical analysis) in a patient with the IgG-κ subtype. We describe the diagnosis, pathogenesis and management of this condition and show the radiological and cytological evidence of the case.

Introduction

Multiple myeloma is a clonal B-cell malignancy characterized by proliferation of plasma cells accumulating mainly in the bone marrow and secreting paraprotein.1 Myeloma accounts for around 1% of all malignancies and UK national statistics for 2006 report an incidence of 6.4 cases per 100,000 annually.2 Mean age at diagnosis is 65 years.

Pleural effusion directly attributable to myeloma is exceedingly rare (1-2% of cases).3 We present a case of pleural effusion arising rapidly in a patient with long-standing multiple myeloma and found to be a myelomatous effusion.

Case Report

A 74-year old man with longstanding IgG kappa myeloma presented with a two week history of increasing dyspnoea on exertion. Blood tests showed a normocytic anemia (Hb: 7.4 g/dL, MCV: 90.1 fl), and renal impairment (urea: 9.2 mmol/L, creatinine: 248 µmol/L). Serum electrophoresis suggested rapid disease progression with an increase in paraprotein from 14 g/L to 50 g/L over a six week period. Chest radiographs showed a large left sided pleural effusion (Figure 1). Diagnostic tap of the effusion produced heavily blood stained fluid with features suggestive of an exudate (pH: 7.62, total protein: 93 g/L, LDH: 506 U/L).

The effusion persisted, necessitating therapeutic drainage which produced four liters of bloodstained fluid. Computed tomography (CT) of the chest was radiologically suggestive of mesothelioma (Figure 2) but cytological analysis of the pleural fluid showed plasma cells consistent with myeloma (Figure 3). Subsequent immunohistochemistry revealed κ light chain restriction and VS38 positivity. A diagnosis of pleural effusion secondary to multiple myeloma was made and the patient was commenced on bortezomib and dexamethasone therapy seven days after first presenting. No further pleural aspiration was necessary from this point and repeat chest radiography (Figure 4) taken seven days after starting treatment showed resolving pleural disease and the patient reported improved symptomatology.

Discussion

Pleural effusion is uncommon in multiple myeloma; it has been suggested that it occurs in approximately 6% of cases. Most of these are not directly due to myelomatous infiltration but to related pathologies such as pulmonary embolus, heart failure and nephrotic syndrome. Effusion directly due to myeloma is said to occur in less than 1% of cases with approximately 80% of these being in IgA disease.3

Involvement of the cavities is an ominous feature in multiple myeloma and thought to represent either a late manifestation in the

Figure 1. Chest radiographs showed a large left sided pleural effusion.

Figure 2. Computed tomography of the chest was radiologically suggestive of mesothelioma.

Figure 3. Cytological analysis of the pleural fluid showed plasma cells consistent with myeloma.

Figure 4. Repeat chest radiography taken seven days after starting treatment showed resolving pleural disease.
natural history of the disease or a feature of the aggressive behavior of myeloma. Diagnosis is based on cytological analysis and immunohistochemical staining; pleural biopsy may also yield a diagnosis of myeloma. Malignant plasma cells within the cytological preparation show a typical basophilic cytoplasm with large, eccentric nuclei and prominent nucleoli.4

Several possible mechanisms are postulated for myelomatous pleural effusion: invasion from adjacent skeletal lesions; extension from chest wall plasmacytomas; tumor infiltration of the pleura; and mediastinal lymph node involvement causing lymphatic obstruction.4,5 Currently, the exact mechanism of pathogenesis remains unknown.

The management is two-stage. First, the effects of the effusion must be alleviated with therapeutic and diagnostic thoracocentesis. Once myelomatous pleural effusion is confirmed on cytology, and/or immunohistochemistry, systemic chemotherapy is required to slow the advance of the disease. There is as yet no consensus as to how best to manage this rapid and aggressive phase of disease but we anecdotally show evidence of a good response to bortezomib and dexamethasone therapy.

We suggest that this case highlights the vital importance of diagnostic clarity, where possible, in finding the underlying etiology of a pleural effusion. Myelomatous pleural effusion carries a poorer prognosis and more work is needed to understand the exact pathogenesis of this condition.

References

The progress of prothrombin time measurement

Juha Horsti
Centre for Laboratory Medicine, Tampere University Hospital, Tampere, Finland

Abstract

Warfarin is the most widely used medicine for oral anticoagulant therapy (OAT). It inhibits the synthesis of coagulation factors II, VII, IX, and X in the liver and results in the production of inactive or partially active versions of these factors. Inactive coagulation factors interfere with prothrombin time measurement (Quick and Owen PT) measuring the sum of coagulation activity and inhibition. The narrow therapeutic range here involves a danger of serious complications and the risk of bleeding or thrombosis. The new-generation PT method can measure coagulation activity and inhibition separately. This new technique promotes patient care and anticoagulant medication (warfarin, dicoumarol) based on coagulation activity in vivo. Both therapy and laboratory controls should be unquestionably accurate and based solely on in vivo coagulation activity. Inactive coagulation factors (inhibition) render measurement, calibration, and harmonization. The use of the new-generation PT method based on measurement of coagulation activity in vivo could develop vitamin K antagonist (VKA) therapy for the marked benefit of patients.

Warfarin

The coumarins or VKAs have been the mainstay of OAT for over 50 years. Warfarin is the most widely used drug for OAT world-wide, and it has a predictable onset and duration of action and excellent bioavailability. The therapy is effective for a variety of clinical indications of anticoagulation. The dosage and the clinical response still vary markedly among patients, depending on genetic inheritance, age, and metabolism. Warfarin is a racemic mixture of two optically active isomers, the R and S forms. It is rapidly absorbed from the gastrointestinal tract (within 90 min) and its half-life is 36-42 h. In the circulation warfarin is bound to plasma proteins (mainly albumin). It accumulates rapidly in the liver, where it is metabolized. Warfarin interferes with the cyclic interconversion of vitamin K and its 2,3 epoxide (vitamin K epoxide) and thus is a VKA. Vitamin K is a cofactor in the carboxylation of vitamin K-dependent coagulation factors. VKA inhibits the synthesis of coagulation factors II, VII, IX, and X in the liver, and they remain partially inactive unless 9 to 13 of the amino terminal γ-carboxyglutamate (Gla) residues are carboxylated to form the Ca2⁺-binding γ-carboxyglutamate (Gla) residues (Figure 1). This carboxylation step renders the coagulation factors functionally active: binding to Ca2⁺ and the phospholipid surface. Therapeutic dosages of warfarin decrease, by 30-50%, the total amount of each vitamin K-dependent coagulation factor synthesized by the liver. The secreted molecules from the liver are under-carboxylated, resulting in diminished biological activity (10-40% of normal). Warfarin treatment reduces the number of Gla residues (normal, 9 to 13) per clotting factor molecule, with a concomitant fall in coagulant activity. When the number of residues decreases from 13 to 9, only 70% of the activity of the clotting factor remains; when one molecule contains six carboxylated residues, only 2% activity is present. The liver excretes both active and inactive coagulation factors to the plasma and both affect International Normalized Ratio (INR) measurement, which possibly has escaped notice in the World Health Organization (WHO) recommendation for the prothrombin time methodology.

History

Discovery of vitamin K antagonists (VKAs) in anticoagulation occurred in the 1920s, when the veterinarian Frank Schofield studied the hemorrhagic disease affecting cattle consuming sweet clover. He ascribed the bleeding to a toxin in the clover. Karl Link and his team studied spoiled sweet clover and, in 1939, extracted dicumarol and identified the hemorrhagic agent, which was the substance in the subsequently was found to have drawbacks by reason of its long half-life. Karl Link synthesized more than 150 anticoagulant compounds and found one particularly active molecule, which was named warfarin (a 4-hydroxy compound) after the patent holder, the Wisconsin Alumni Research Foundation. Professor Paul Owen found factor V and developed a new PT method' subsequent to the “Quick method” to overcome its drawbacks. The Owen reagent (combined thromboplastin reagent, Thrombotest) is used mainly in the Nordic countries, Benelux, and Japan. By reason of its different reagent composition, the Quick technique is sensitive to the coagulation factors fibrinogen, II, V, VII, and X, while the Owen technique is affected by deficiencies in factors II, VII, and X. The Quick method measures factor V and fibrinogen, which are not dependent on warfarin therapy, and this constitutes a drawback for OAT.

International Normalized Ratio

The calculation formula is for: INR = (sample/(sample + normal))/ISI, where ISI is the International Sensitivity Index. When the ISI is near 1.0, the reagent is sensitive and ISI has little meaning in the INR calculation. The Quick and Owen PT methods are the most common and generally accepted means of monitoring VKA therapy. A comparison between the PT methods has been published in a recent article and the Owen PT was superior. WHO recommends the use of the INR to harmonize PT results and therapeutic ranges globally both for patient care in clinical practice and in the scientific literature, as the units used formerly proved inadequate for international communication. The challenge is for global clinical laboratories to harmonize INR testing further to a level where INR results are consistent regardless of the methods used.

The use of the INR system still involves difficulties with sample citrate concentration, different reagents and thromboplastins, and instruments, and with ISI and "local ISI" calibration to harmonize results. Horsti and colleagues measured 150 samples from patients on oral anticoagulation using seven commercial reagents and four different calibrator kits. Agreement between INR results was poor.
ISI calibration

The reagent manufacturer informs the ISI value or the laboratory can measure the ISI with an ISI calibration kit (local calibration) for a PT reagent lot. The meaning of ISI as power in the INR calculation equation is displayed in the previous section.

First, the aim in the original recommendation\(^29\) was to harmonize INR results by calibrating reagent ISI values with Human Combined, which was the primary reference preparation (IRP code 67/40). Thromboplastins from different sources (human brain, rabbit brain, rabbit lung, and ox brain) yield quite different levels of PT. The hierarchy of reference thromboplastin preparations was presented by van den Besselaar and associates.\(^21\) As the WHO calibration procedure was complex and demanding in the second stage, the recommendation for ISI calibration was local calibration using certified lyophilized plasmas.\(^31-37\) A third possible approach for calibration was presented by van den Besselaar and group. This alternative means of determining the ISI involves the use of freshly pooled plasmas from 20 normal individuals and 60 patients receiving coumarin (OAT). Such numbers of samples are necessary to obtain a precise calibration line for ISI calculation. Freshly pooled plasma can be used to determine reagent or instrument ISI with acceptable precision, or as good a result as with the WHO calibration model.\(^29\)

Poller and colleagues (European Concerted Action on Anticoagulation, ECAA) have compared local ISI calibration and “Direct INR” in the correction for locally reported INRs. For local INR correction (harmonization) they used seven normal plasmas and 20 from patients on warfarin. The results were variable.\(^39\) It is clear that plasmas from patients on warfarin therapy always involve varying amounts of inactive coagulation factors, depending on the level of anticoagulation and individual patient characteristics (see section on Warfarin).

All these former calibration models involve the principle that the calibrator contains an average amount of inactive coagulation factors (inhibition), which means an average correction for patient INR results. Is the average correction of inactive coagulation factors appropriate for individual patient samples in a measuring range from 1 to 5 INR? Is it a fact that calibrators, according to biochemical principles, contain inactive coagulation factors (inhibition)? Do different reagents behave identically to active and inactive coagulation factors?\(^22\) In our earlier studies we measured inactive coagulation factors (inhibition) in four different kits and noted conspicuous variation in inhibition.\(^40\) ISI calibration should be based on normal plasma and normal plasma dilutions without inactive coagulation factors. In principle, calibrators should not contain inhibitory coagulation factors, which cause error in calibration.\(^6\)

The new-generation prothrombin time method

Today the Quick and Owren PT methods measure the sum of active coagulation factors (FII, FVII, FX) and inhibition by their inactive coagulation factor counterparts. The new-generation PT method can measure separately both active coagulation factors (FII, FVII, FX) and the inhibition caused by inactive or partially inactive coagulation factors.\(^31\) Inhibition varies markedly between individual patient samples, depending on the medication and metabolism. Correction of inhibition must be made individually for each patient sample.\(^41\) In this method two measurements for one sample are needed and the cost per sample is twice as much. The average correction does not suffice for accurate INR results. In addition, the number of inactive coagulation factors varies markedly in different calibrator kits (manufactured or local). This means difficulties in calibration and harmonization of different reagents.\(^29\) The harmonization of INR results using different PT methods and reagents is problematic if attempted according to the WHO recommendation.\(^29\) Measuring only the active coagulation factors without inhibition harmonization between different reagents succeeds well.\(^31\)

Conclusions

New medications for OAT have been developed over a number of years and anticipated...
without laboratory test control in an effort to displace warfarin medication. The new medicines, however, have not proved their superiority over warfarin. The new molecules are too expensive for global use or involve serious side-effects and possibly will never be as popular as warfarin, which is an old and cheap means widely used and accepted globally. The only drawback with warfarin medication is regular laboratory control. It would be important to develop warfarin therapy with greater attention to laboratory control, which helps patient care. Different reagents and thromboplastins react variably with inactive coagulation factors and cause difficulties in calibration using patient plasmas that contain inhibiting coagulation factors. Data on inactive coagulation factors are individual and should be corrected individually. Every new anticoagulant therapy patient should be tested for inhibition at the commencement of therapy.

The accuracy and harmonization of patient INR results for different reagents is of the utmost importance for scientific publications, medication, and patient care. Today’s measuring principle is taking the sum of active coagulation factors (FII, FVII, FX), and inhibition of inactive coagulation factors is not a satisfactory approach from the standpoint of accurate patient care. Active coagulation factors in vitro are responsible for thrombosis and bleeding. The errors in INRs are too great, which in many ways affects the success of medication and patient well-being, also using the same calibration (local calibration). The new-generation PT offers possibilities of more accurate INR results and patient care based on control of active coagulation factors. The inhibition renders measurement, calibration, and harmonization. The therapeutic INR ranges guiding anticoagulant therapy using “old methods” are based on the principle that both calibrators and patient samples have inactive coagulation factors (inhibition) on average, which compensate for each other in the final INR result. Thus the therapeutic ranges are available for the new-generation PT method. The therapeutic range lies between thrombosis and bleeding, and complications are serious and general. The medication and care of OAT patients must be rendered better and safer using a more sensitive PT test.

References


Introduction

The presence of Hb Barts in blood at birth is a finding in α-thalassemia trait, intermedia (HbH disease) and major (hydrops fetalis). \(^\text{12}\) Diagnosis of α-thalassemia major is not a problem and it is incompatible with life as most of the hemoglobin will be HbA and HbBarts, usually more than 15%. \(^\text{4}\) This condition is easy to diagnose later in life, even if not investigated at birth, due to the presence of HbH. \(^\text{4}\)

The problem lies with α-thalassemia trait, single or double gene deletion types. Most of these cases have normal HbA2 levels if checked after the age of six months.\(^\text{6,7,8}\) though red cell indices are thalassemic. This group, which makes up the bulk of gene carriers, will be difficult to confirm by Hb electrophoresis (HBEp) or by HPLC in childhood and adult life. If one can check HBEp or HPLC at birth then presence of Hb Barts would put the diagnosis on solid grounds. Hb Barts disappears a few weeks after birth.\(^\text{1}\)

It is too expensive to perform HPLC or HBEp for all neonates, even in areas where frequency of the α-thalassemia gene is high. So, if we can predict, with cheap tests, the presence of α-thalassemia then Hb separation can be performed for the highly probable cases only. This issue was addressed by work carried out in Thailand,\(^\text{4}\) and in Jeddah, Saudi Arabia a few years ago. \(^\text{1}\) MCV below 90 and MCH below 30 were found to be of very high predictive value in the Jeddah study.

There was a need to repeat the study in another setting, where thalassemics are of variable ethnic groups, like in Dubai. α-thalassemia is present in the UAE nationals and residents of Iranian, Arab, Pakistani and Filipino origins in Dubai and it was expected that neonates in such a population would make a good sample for repetition of the study and confirmation of the validity of using the MCV and MCH as predictive parameters in individuals of various ethnic groups.

Materials and Methods

All full blood counts (FBC) performed for neonates were checked. Counts were performed using a Coulter machine model LH7D1 (Beckman Coulter, Brea, Ca, USA). Thirty-four consecutive neonates, with MCV below 90 fL were checked by HPLC, using Waters 2695 machine (Milford, Ma, USA), and short β-thalassemia program, to look for Hb Barts band.

Blood films of all babies included in the study were checked by the hematopathologist for red cell morphology. FBCs had not been ordered for these babies because of any suspicion of thalassemia. They were random and judged to be necessary by the attending neonatologists for various reasons.

As a control group, 26 neonates, who had MCV between 90 and 95 fL were also checked by HPLC. Again, these were not selected and all consecutive cases with such an MCV level were included into the control group. It took eight months to collect the entire test and control cases because only a minority of neonates in Dubai have blood counts checked.

Results

All individuals in the test group had MCV below 90 fL (mean 85.35, range 77-88.9). MCH was below 30 in 33/34 patients (97%). One patient (3%) had an MCH of 31.1 pg. Mean MCH was 27.82 and range 25.1-31.1. Hb Barts was demonstrated in all the 34 cases (100%). It ranged from a trace (less than

<table>
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<th>≥90</th>
<th>&lt;30</th>
<th>≥30</th>
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<td>Negative</td>
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<td>0</td>
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<th>Hb Barts</th>
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<th>≥90</th>
<th>&lt;30</th>
<th>≥30</th>
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</table>
extend the MCV level to 95, whether or not the baby is born premature. In such cases one can expect, with high confidence, to find α-thalassemia.3,5,11 The work carried out in Jeddah and in Dubai a MCV cut-off value of 95 was used to identify a small proportion of these individuals.5,10,11 However, there is no clear demarcation of the level of MCV and MCH where one can expect, with high confidence, to find α-thalassemia.11,12 The work carried out in Thailand3 took the MCV cutoff value of 95. In our experience, in both works performed in Jeddah and in Dubai a MCV cut-off value of 95 will give a lot of negative cases and make the need for HPLC/HBEP much higher, thus increasing the cost while one of the objectives of this scheme is to lower costs.

The advantage of using MCV and MCH levels is that these values are easily available from a simple automated blood count (FBC). If one can prove that there are limit levels for these two parameters which carry a high probability of over 95% that the case is confirmed from this study that if MCV is below 30 and MCH below 12.5 and later proved to have Hb H disease. MCV in the control group ranged from 90.1 to 94.8 fl (mean 92.27) and MCH from 28.7 to 31.7 (mean 30.33). Hb Barts was demonstrated in 11 cases (42.3%). It ranged from 2-6% in those cases. Fifteen cases (57.6%) were negative for Hb Barts. Analyzing the 11 Barts-positive cases further showed that only one baby (3.8%) out of the 26 controls and positive Barts had MCH above 30 and was full term (3 of them had MCH below 30).

Blood films of all babies included in the test group showed numerous target cells and microcytosis, but no signs of active hemolysis.

Discussion

After the work carried out in Jeddah, Saudi Arabia,5 where the population is basically local with a low expatriate mix, as well as some variation in the origin of the local population itself, it was thought useful to the medical literature to repeat the study at another site to confirm the remarkable results of the original study. Medical literature does mention that α-thalassemia trait individuals are born with microcytosis.10,11 However, there is no clear demarcation of the level of MCV and MCH where one can expect, with high confidence, to find α-thalassemia.11,12 The work carried out in Thailand3 took the MCV cut-off value of 95. In our experience, in both works performed in Jeddah and in Dubai a MCV cut-off value of 95 will give a lot of negative cases and make the need for HPLC/HBEP much higher, thus increasing the cost while one of the objectives of this scheme is to lower costs.

The advantage of using MCV and MCH levels is that these values are easily available from a simple automated blood count (FBC). If one can prove that there are limit levels for these two parameters which carry a high probability of α-thalassemia trait and HbH disease, then there will be a small proportion of patients for whom one needs to perform Hb separation procedure, by alkaline electrophoresis or HPLC, to demonstrate Hb Barts, and thus prove α-thalassemia presence. It is confirmed from this study that if MCV is below 90 and/or MCH is below 30 there is a high probability of over 95% that the case is α-thalassemia trait. MCV may be above 90 if the baby is born premature. In such cases one can extend the MCV level to 95, whether or not MCH is below 30. It is noteworthy that Hb Barts is not only a diagnostic finding for α-thalassemia, but it tends to disappear a few weeks after birth.11,12 Trying to confirm β-thalassemia trait at a later age, when suspicion is raised by low red cell indices leading to erythrocytosis, hematologists first resort to HBEP or HPLC, looking for low HbA2. However, low HbA2 is not a common finding in this condition.11,12 Also, low Hb A2 is found in other conditions, like β-thalassemia. Iron deficiency anemia may also lower HbA2 to below normal level.11,12 Failing to confirm the condition by this routine test, as in the majority of cases, one can try molecular studies on the α-polypeptide gene to look for deletions or mutations known to lead to α-thalassemia trait. These deletions and mutations are so numerous11,12 that, in practice, laboratories only try the common local deletions or mutations, using the proper probes for them, if these are known from previous studies. In a mixed population the task will be difficult, as one can imagine. The cost of these molecular tests is certainly higher than Hb separation studies. In parts of the world where α-thalassemia gene is of high frequency, the cost will be enormous if one wants to diagnose all carriers. It should be remembered that most countries harboring a high frequency for the gene are also poor in resources.11 The simple strategy we are recommending, proven by the two studies in two different locations, is valid and is recommended by the authors for WHO and local public health schemes to establish the diagnosis and gene frequency. Further studies are being considered to try to split populations with one, two and three gene deletions/mutations from levels of MCV and MCH caused by them and the HPLC performed as a result of obtaining the low MCV and MCH figures. The simple investigational scheme that we suggest to be followed for the α-thalassemia screening of neonates is shown in Figure 1.


Best practices for transfusion for patients with sickle cell disease

Ted Wun,1 Kathryn Hassell2
1UC Davis School of Medicine, Sacramento; 2University of Colorado at Denver, USA

Abstract

The β-globin gene mutation in sickle cell anemia results in anemia and repeated bouts of vascular occlusion. The cumulative effect of these vasoocclusive events is progressive damage to many organs including the kidneys, lungs, and brain. The transfusion of red blood cells (RBC) can ameliorate many of these complications, but can be associated with both acute and chronic complications, including iron overload. The objective of the Best Practices in Transfusion Medicine for Patients with Sickle Cell Disease (SCD) Conference was to review the available published evidence and clinical experience surrounding the use of RBC transfusions for sickle cell disease by a panel of experts. The expert panel developed explicit clinical guidelines for the use of RBC in SCD patients. The panel also made recommendations for further research. A set of guidelines were produced for dissemination to pertinent stakeholders. If implemented, these clinical pathways have the potential to optimize the use of red blood cell transfusions in SCD.

Introduction

Sickle cell anemia was the first recognized “molecular disease”. A single DNA base mutation, leading to a single amino acid substitution (Glu to Val), results in hemoglobin S (HbS), the major hemoglobin defect, and a myriad of clinical manifestations.1 Acute clinical manifestations include painful crisis, acute chest syndrome, stroke, and priapism. Irreversible major organ damage occurs to the brain, heart, lungs, kidneys, eyes, and femoral heads. Although anemia accounts for some of the morbidity of sickle cell disease, vascular occlusion underlies most of the acute and chronic complications.1 The SCD phenotype is predominately expressed in those with homozygous S (HbSS), or compound heterozygotes of HbS and HbC (HbSC) or HbS and β-thalassemia (HbS/B-thal or HbS/Sβ-thal).

There is no doubt that both acute and chronic RBC transfusions, both in the acute and chronic setting, can ameliorate many of the complications of sickle cell disease.2 Some transfusion practices, such as perioperative transfusion,3 transfusion during pregnancy,4 and chronic transfusion to prevent stroke in children found to be high-risk from transcranial doppler (TCD) screening and/or because of previous stroke,5 are based on prospective randomized studies. Other practices, such as transfusion for prolonged painful episodes, exchange transfusion for acute chest syndrome, and RBC transfusion for those with pulmonary hypertension, are based on less firm evidence.6 Barriers to chronic transfusion therapy include patient and family reluctance, inconvenience, intravenous access issues, alloimmunization, red cell availability, infectious disease complications, immune modulation, and iron overload.4

Based on surveys from the Sickle Cell Adult Patient Network (SCAPN), whereas transfusion practice for patients with pregnancies is fairly standard, it varies considerably in adults with ischemic stroke (K. Hassell, unpublished communication).

It was recognized that many practices and recommendations regarding transfusion of RBC for patients with SCD were not evidenced based. Nonetheless, it was felt important to highlight those that did have a basis in prospective, randomized trials, and to catalog practices that were in common use but not evidenced based. At a minimum, such a catalog could serve as a basis for critically examining current practices and setting research priorities for future studies. The overall purpose of the conference was to provide succinct best practices guidelines for transfusion that had the potential to improve care.7

Materials and Methods

This conference was conceived to be part of a series of five best practices guidelines focusing on the care of adult patients with SCD. This conference was not a “consensus” conference, nor was it an exhaustive, meta-analyses or Cochrane-type review of the literature. Rather, it was a focused literature review with key citations as determined by experts in the field. For a more comprehensive overview, the reader is directed to several excellent reviews on this topic.8,9,10 Several months prior to the meeting, the conference coordinator (TW) asked each invited expert (Table 1) to forward succinct recommendations for each assigned topic and provide key citations. These served as the basis for the agenda.

The conference took place September, 2007 immediately preceding the annual Sickle Cell Clinical Research Meetings and was open to the public. Each expert was asked to present her or his recommendations and assign a strength based on a simplified scoring system of Strong, Moderate, or Uncertain (Table 2) that paralleled ones used in other guideline recommendations.11 At the end of each presentation, comment was invited from the audience and other panel members. The final recommendations were developed by consensus at the end of the conference with audience input. Panel members adhered to several principles while developing their recommendations. First, in keeping with the theme, best practices, as opposed to standard or practical ones, were to be recommended. The bar was to be set high to promote what was felt to be optimal medical care. Second, recommendations were to be evidence-based if possible, but could also be based on expert opinion. This was in recognition that very little available data was truly evidenced-based. In the absence of data specific to adults, discussants were asked to extrapolate from data in children. Finally, panel members were asked to be as prescriptive as possible, without being dogmatic.

Results

A set of guidelines were produced for dissemination to pertinent stakeholders.

Recommendation #1: indications for acute red blood cell transfusions

Transfusion is indicated for the therapy of the acute clinical situations detailed in Table 3. This recommendation was strong for acute chest syndrome (ACS) with hypoxemia or worsening respiratory distress,12 ischemic stroke without or without post-infarct bleeding,13 splenic and hepatic sequestration with worsening anemia, aplastic crisis with worsening ane-
mia, symptomatic acute blood loss, and multiorgan failure.\textsuperscript{22} It was acknowledged that there is neither a universal definition of acute chest syndrome, nor a system for grading the severity of ACS.\textsuperscript{14\textendash}16 Nonetheless, all panel members agreed that hypoxemia requiring supplemental oxygen and/or worsening tachypnea were indications. It was also recognized that the term “significant decrease from baseline” in regards to splenic and hepatic sequestration is subjective. This determination is best made by clinicians under specific clinical circumstances. For isolated intracerebral hemorrhage, transfusions are of uncertain benefit and, anecdotally, have exacerbated central nervous system (CNS) bleeding. Nonetheless, in cases where there was an infarctive component to the bleed, some panel members felt that transfusion might be useful. Panel members were in more agreement about transfusion for transient ischemic attacks (TIA), but there are some data suggesting that transfusions are not effective at prevention.\textsuperscript{23} However, in the absence of clinical trials, this recommendation was graded as only moderate. Silent cerebral infarcts (SCI) were not addressed at this conference, but there are some data to suggest that transfusion may lower the incidence of SCI as defined by MRI.\textsuperscript{24} However, as these data were generated in children who would be considered candidates for transfusion therapy regardless (because of high risk TCD findings), a separate recommendation may not be necessary. The indications for red blood cell transfusion for severe infections and persistent priapism are based on less convincing anecdotes and are thus uncertain.\textsuperscript{25} The association of sickle cell disease, priapism, exchange transfusion, and neurological events (ASPEN syndrome) was also of concern regarding transfusion therapy for priapism.\textsuperscript{26,27} Because the ASPEN syndrome is based largely on case reports, there was insufficient evidence to consider priapism a contraindication to transfusion. A recent report suggested that exchange transfusion was safe and efficacious if the resulting hemoglobin level was <11 g/dL,\textsuperscript{28} suggests that ASPEN syndrome results from increased whole blood viscosity.

**Recommendation #2: red cell transfusion is not indicated for the therapy of uncomplicated acute painful episodes regardless of duration**

The panel discussed that RBC transfusion during otherwise typical painful episodes was common in both community and academic settings but there was little to no evidence to support this practice.\textsuperscript{27} The term uncomplicated in this context means without the indications for acute transfusion stated in recommendation #1. The panel felt that this practice should be discouraged, therefore warranted a separate recommendation to emphasize the point.

**Recommendation #3: acute red cell transfusion is not indicated for a decrease in hemoglobin from baseline not associated with symptoms of anemia**

In a certain sense, this recommendation is in concert with #2. Patients with SCD have variable severity of chronic anemia. Physiological compensation includes high cardiac output and increased plasma volume. The practice of routine transfusion for an arbitrary level of hemoglobin should be discouraged in SCD patients and all patients. The panel emphasized that patients with chronic anemia may be more sensitive to volume challenge and prone to develop symptomatic volume over-load. Whether a patient tolerates a specific level of hemoglobin depends on the clinical circumstances (e.g., infection, rapidity of decline, co-morbid conditions) and the determination of whether the anemia is “symptomatic” is somewhat subjective. This recommendation is dependent on sound clinical judgment.

**Recommendation #4: prophylactic red cell transfusions**

Red cell transfusions can be used in the prevention of sickle cell complications. The panel felt that these should be strongly recommended for high risk surgical procedures,\textsuperscript{24,25} secondary prevention of stroke, and primary prevention of stroke in those children identified as high risk by TCD examination.\textsuperscript{26} In patients requiring general anesthesia with evidence of cardiac, pulmonary, renal, or hepatic dysfunction, many panelists and attendees also felt red cell transfusions were indicated. The panel discussed general anesthesia is likely not the issue, but rather procedure times of 45-60 minutes or longer place patients at higher risk for complications, predominantly pulmonary. There was disagreement as to what constituted a high risk surgical procedure. There was agreement that open thoracic and vascular procedures were high risk, but hip and knee replacement might be considered moderate risk (similar to cholecystectomy). Abdominal procedures may also be associated with increased risk, particularly in patients with HbSC disease.\textsuperscript{27} Anecdotal experience suggests that prophylactic transfusions can reduce the frequency of painful episodes in patients refractory to hydroxyurea, but only as part of a comprehensive pain program.

**Recommendation #5: chronic transfusion may be useful in stabilizing or reversing certain end-organ dysfunction**

These include pulmonary hypertension, congestive heart failure, and renal dysfunction. However, this recommendation is almost entirely based on personal experience and/or anecdotal reports. Many participants observed...
that aggressive local therapy was the most effective treatment for non-healing leg ulcers. As the sickle cell population ages and develops more chronic end-organ dysfunction, this will be an important area for research.

Recommendation #6: type of red cell product

The red blood cell products used for transfusion in patients with SCD should all be leukocyte-reduced, sickle hemoglobin negative, matched for at least the red cell antigens Cc, Ee, and K and any antigens for which the patients has developed alloantibodies (past or present). Alloimmunization to red cell antigens is a common occurrence in transfused SCD patients.27 Limited phenotype-matching has been shown to decrease significantly the rate of alloimmunization amongst non-alloimmunized sickle cell patients.28 Recent studies showed that limited phenotype-matching is practiced by a minority of transfusion medicine services surveyed in North America.30 Discussion centered on the fact that not all cellular blood products in the US are pre-storage leukocyte reduced. While this is the standard in the European Union and the United Kingdom, only about 70% of blood banks in the United States routinely stock pre-storage leukocyte reduced products. Not all transfusion medicine services verify that blood being transfused to a SCD patient is sickle hemoglobin negative. In areas where there is a large African American blood donor population, this issue becomes more relevant.

Recommendation #7: target hemoglobin level

When transfusing red blood cells for acute indications, the target hemoglobin and hematocrit level should not exceed 10 g/dL and 30%, respectively. The panel and audience believed that there were sufficient rheological data and rationale to avoid higher levels to prevent increases in whole blood viscosity.31-33 In addition, there is some clinical evidence suggesting that acute clinical deterioration occurs when whole blood viscosity is suddenly increased (ASPEN Syndrome noted above). The panel acknowledged that much of the data were generated in vitro; nonetheless, this recommendation was strong.

Recommendation #8: target HbA level

For acute exchange transfusions, a level of HbA% >70% should be targeted. It was acknowledged that this has never been rigorously studied. Establishing a target in terms of the percentage of HbA rather than HbS was more sensible for compound heterozygous patients. For example, it is simpler to target raising the HbA to 70% in a patient with HbSC than to target a

Table 3. Recommendations.

<table>
<thead>
<tr>
<th>Recommendation</th>
<th>Description</th>
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<tr>
<td>#1</td>
<td>Acute complications requiring acute red cell transfusion</td>
</tr>
<tr>
<td>#2</td>
<td>Acute red cell transfusion is not indicated for the therapy of uncomplicated painful episodes regardless of duration (Strong)</td>
</tr>
<tr>
<td>#3</td>
<td>Acute red cell transfusion is not indicated for a decrease in hemoglobin from baseline not associated with symptoms of anemia (Strong)</td>
</tr>
<tr>
<td>#4</td>
<td>Prophylactic red cell transfusions are indicated for</td>
</tr>
<tr>
<td>#5</td>
<td>Chronic transfusion may be useful in stabilizing or reversing</td>
</tr>
<tr>
<td>#6</td>
<td>The red blood cells given to patients with sickle cell disease should be</td>
</tr>
<tr>
<td>#7</td>
<td>For acute sickle cell complications, the dose of red cells should be calculated to result in a Hb ≤10 g/dL or ≤Hct 30% (Strong)</td>
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<tr>
<td>#8</td>
<td>The goal of exchange transfusion for acute indications is to achieve a HbA &gt; 70% on post-transfusion hemoglobin fractionation (Strong)</td>
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<tr>
<td>#9</td>
<td>Exchange transfusion is preferred over simple transfusion in adults for</td>
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<tr>
<td>#10</td>
<td>Every patient with sickle cell disease should be vaccinated for hepatitis A/B (Strong)</td>
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and extent of this assessment.34 The panel noted that some published recommendations are an extrapolation from the experience with β-thalassemia patients whose iron burden is significantly higher based on pathophysiology of their disease.35,36 Despite limited evidence, the consensus of the panel is that the hepatic storage iron concentration should be maintained below a threshold of 15 mg Fe/g liver, dry weight, to avoid progression to liver fibrosis.

Recommendation #9: simple vs exchange transfusion

There are few data comparing simple to exchange transfusion. Exchange was preferred strongly over simple transfusion for the treatment of severe acute chest syndrome, stroke, and multi-organ failure. A multi-center, randomized study showed exchange transfusion to be non-superior to simple transfusion in the perioperative setting.34 However, the panel endorsed the use of exchange as preferable in advance of high risk operations. There was no clarity as to which modality was preferable in the setting of acute priapism and use of hyperosmolar contrast agents, with the latter just to be avoided with the availability of iso-osmotic contrast agents.

Recommendation #10: vaccination

Every sickle cell patient should be vaccinated for hepatitis A and B as early as possible to prevent post-transfusion infection. This was non-controversial and strong.

Recommendation #11: iron assessment

There was consensus that chronically transfused sickle cell disease patients should be regularly assessed for iron overload and offered chelation therapy to decrease the risk of iron related toxicity. There was considerable discussion regarding the number of lifetime transfusions that should trigger an assessment of iron stores, and the optimal methods and extent of this assessment.34 The panel noted that some published recommendations are an extrapolation from the experience with β-thalassemia patients whose iron burden is significantly higher based on pathophysiology of their disease.35,36 Despite limited evidence, the consensus of the panel is that the hepatic storage iron concentration should be maintained below a threshold of 15 mg Fe/g liver, dry weight, to avoid progression to liver fibrosis and a target range of 3 to 7 mg Fe/g liver.

Recommendation #12

Every patient with sickle cell disease should have a portable comprehensive transfusion record, including phenotype, type and number of transfusions, alloantibody status and adverse events. Strongly advocated by the patients present, this again would ensure timely provision of appropriate and safer red cell transfusions.

Recommendation #13: information sharing

There should be a system for sharing patient information on RBC phenotype, and alloantibody status and autotransfusions among regional blood providers. This recommendation was stressed by the patients and family members in attendance.

Conclusions

If implemented, these clinical pathways have the potential to optimize the use of red blood cell transfusions in SCD.

References


