Differential Effects of Somatostatin on TNF Receptors and Apoptosis in Hepatocellular Carcinoma Cell Lines

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Abstract: The anti-tumoral activity of somatostatin has been demonstrated in both animal experiments and human tumors. Clinical trials have reported conflicting results. We therefore hypothesized that somatostatin might have different effects in various hepatocellular carcinoma cells. Their clarification would possibly allow for the better selection of patients suitable for the optimal treatment results. We studied the mRNA and protein expression of TNF receptors and the TNFa-induced apoptosis using the HepG2 and the Hep3B human hepatocellular carcinoma cells after incubation with the somatostatin analog octreotide. RT-PCR, Western blot, and parameters associated with apoptosis (NF-kB nuclear translocation, P65 Ser536 and P65 Ser468 phosphorylation, DNA fragmentation) were assessed. Only TNFR1 was constitutively present in the two cell lines. Octreotide incubation led to an earlier reduction in TNFR1 mRNA and protein in HepG2 compared to Hep3B cells (1 h and 6–12 h, respectively). NF-kB translocation to the nucleus was induced by TNFa and was more prominent in Hep3B. Translocation was unaffected by octreotide. Serine phosphorylation was significantly induced by TNFa and was more evident in the Hep3B cells. TNFa-induced Ser536 phosphorylation was inhibited by octreotide only in the HepG2 cells. DNA fragmentation was not influenced by either octreotide or TNFa in the HepG2 cells, but TNFa induced fragmentation in the Hep3B cells (1.8-fold increase) verified by the TUNEL index (43 compared to 19 for the HepG2 cells). Octreotide and TNFa co-incubation induced apoptosis in the HepG2 cells (1.7-fold increase compared to controls) but inhibited apoptosis in the Hep3B cells. We conclude that: (1) octreotide reduced TNFR1 receptor expression in both cell lines, (2) parameters of apoptosis were differentially affected by octreotide in the two cell lines, and (3) this might be a partial explanation for the conflicting results of somatostatin analog treatment in human hepatocellular carcinoma trials.

Keywords: octreotide; apoptosis; TNF receptors; NF-kB; hepatocellular carcinoma cell lines

1. Introduction

Somatostatin has been used in the treatment of various neoplasms. Evidence from both neuroendocrine and non-hormone-producing tumors indicate that the synthetic somatostatin analog octreotide may be a promising anti-cancer therapeutic agent [1,2] including hepatocellular carcinoma (HCC). HCC is the seventh most common malignancy and the second cause of cancer mortality worldwide [3], but treatment of advanced HCC is still dismal [4,5]. Octreotide is a synthetic version of the natural hormone somatostatin that resembles the native polypeptide in its activity, which is stable for a longer period of time. We have previously shown that octreotide has a direct anti-proliferative effect on...
human hepatoma cells HepG2 [6]. It was first tested in patients with unresectable HCC in a randomized controlled study and was found to significantly improve survival [7]. This trial was followed by several trials using somatostatin analogs in inoperable HCC with conflicting results. Nevertheless, a critical evaluation of the literature justifies the need for better-designed studies and further investigation of this issue. The reason for these conflicting results is not clear. Activation of somatostatin receptor 2 (sst2) induces apoptosis, but there are several other octreotide actions that may be responsible for its anti-tumoral activity [8] including the TNFa pathway.

Tumor necrosis factor alpha (TNFa) has a critical role in hepatic homeostasis and physiology since it is implicated in either hepatocellular death or cell proliferation, depending on the liver microenvironment [9]. TNFa is a trimer of 157 amino acids binding to two receptors: TNF receptor 1 (TNFR1) of 55 kilodaltons (kDa) and TNF receptor 2 (TNFR2) of 75 kDa [10]. TNFR1 is constitutively present on most cell types, while the presence of TNFR2 depends on a number of stimulatory agents [11]. TNFR1 binds soluble TNFa [12] and TNFR2 recognizes membrane-anchored TNFa [13]. TNFR1 is involved in apoptosis as TNRR2 lacks a death domain [14].

The nuclear factor-kappa B (NF-kB) family of factors is involved in cellular proliferation, apoptosis, and inflammation. It consists of five transcription proteins: p65 (RelA), RelB, c-Rel, p105/p50, and p100/p52. p50 and p52 are produced by the proteolysis of p105 and p100. Upon activation, they all form dimers and then translocate into the nucleus and activate the transcription of target genes [15]. The NF-kB is activated either through the canonical or non-canonical pathways. TNFa, lipopolysaccharides, and interleukin-1 activate NF-kB by using the canonical pathway and induce genes implicated in inflammation and cell survival. On the other hand, TNF superfamily members such as the receptor activated NF-kB ligand (RANKL) and lymphotoxin β induce genes implicated in the tumor microenvironment through the non-canonical pathway [16,17]. The NF-kB pathway usually, but not always, protects liver cells from apoptosis [18,19]. Evasion of apoptosis is a characteristic of cancer. This may be achieved through a crosstalk between the tumor suppressor p53 and the NF-kB pathway. Inflammatory stimuli such as TNFa activate NF-kB, leading to the induction of anti-apoptotic genes that inhibit the pro-apoptotic function of p53. On the other hand, p53 and NF-kB co-operate when some inducers of NF-kB suppress anti-apoptotic genes and induce pro-apoptotic genes. The final effect probably depends on the cell type and the inducing stimulus for both p53 and NF-kB [17,20,21]. The NF-kB signaling pathway is activated in various colorectal cancer cell lines regulating anti-apoptotic genes. Inhibition of these genes increases apoptosis [22]. It should be noted that DNA damage leads to a transient anti-apoptotic first phase of NF-kB activation followed by a persistent inflammatory second phase [23]. In hepatocellular carcinoma (HCC) pathogenesis, a dual role for TNFa-induced liver cell NF-kB activation has been reported. A beneficial cytoprotection occurs in the early stages of liver carcinogenesis inhibiting apoptotic death and the subsequent compensatory hyperproliferation of mutated cancer-prone hepatocytes [24]. However, at later stages it favors malignant transformation by offering survival benefit to hepatocytes [25]. The p50/p65 heterodimer [26], which is often used as a synonymous for NF-kB, is the family member most extensively studied. In resting cells, NF-kB is an inactive cytoplasmic protein attached to an inhibitory kB (IkB) factor, mostly IkBα. In response to TNFa and other inflammatory stimuli, NF-kB activation occurs, releasing NF-kB for nuclear translocation, where it mediates the expression of certain pro-survival genes [27,28]. In addition to nuclear translocation, the p65 subunit of NF-kB must undergo several post-translational modifications including phosphorylation at serine 536 to be fully activated. The phosphorylation of NF-κBp65 favors hepatocellular carcinogenesis, responding to the inflammation-mediated overproduction of β-arrestin1 (ARRB1). Suppression of the phosphorylation of NF-κBp65 inhibits hepatocellular carcinogenesis [29]. Other factors may also modulate the final effect of NF-kB on cell survival. In this respect, glycosylation is a very important factor. Glycans control several cellular events including apoptosis. O-GlcNAcylation of NF-kB is a critical molecular mechanism that
regulates cell proliferation and survival [30–32]. Sialylation also regulates the function of TNFR1. A2-6-linked sialic acid residues of N-glycans regulate TNFa-induced apoptosis and their removal enhances apoptosis [33,34].

To explain the differences in clinical trials, we hypothesized that somatostatin might have different effects in various hepatocellular carcinoma cell lines. We therefore studied the effect of octreotide on TNFa signaling in the HepG2 and Hep3B human hepatocellular carcinoma cell lines. In particular, we assessed the influence of octreotide on TNFRs expression and NF-kB activation, and we further tested for potential modifications of the apoptotic response of cells to TNFa caused by octreotide. We kept the name TNFa instead of the recently proposed TNF as the majority of authors still use this term [35].

2. Materials and Methods

2.1. Materials

Gibco BRL Life Technologies (Paisley, UK) provided the cell culture reagents. Human recombinant TNFa was bought from R&D Systems (Abingdon, UK), rabbit polyclonal anti-TNFR1 antibody from Abcam (Cambridge, UK), mouse monoclonal anti-β-actin antibody from Santa Cruz Biotechnology (California, CA, USA), and octreotide from Novartis (Basel, Switzerland). Invitrogen (Carlsbad, California, CA, USA) provided the TRIzol, Oligo (dT)12–18 primer. Superscript II, RT buffers, and 100 bp DNA ladder RNAsin were bought from Promega (Southampton, UK). Roche (Basel, Switzerland) provided the deoxyribonucleotide triphosphates (dNTPs), polymerase chain reaction (PCR) buffers, DNAse I, and expand polymerase. Primers for the reverse transcription (RT)-PCR studies were synthesized by VBC Genomics (Vienna, Austria). All other chemicals were purchased from either Sigma-Aldrich (Steinheim, Germany) or Merck (Darmstadt, Germany).

2.2. Culture of Hepatocellular Carcinoma Cell Lines

Hepatocellular carcinoma cells HepG2 and Hep3B were purchased from DSMZ (Braunschweig, Germany). The cervical adenocarcinoma cell line HeLa was a gift from Dr PA Theodoropoulos (Medical School, University of Crete, Greece). Cell lines were kept at 37 °C, in a humidified atmosphere containing 5% CO2 and cultured in RPMI 1640, Minimum Essential Medium (MEM) plus 1% non-essential amino acids, or Dulbecco’s modified Eagle’s medium (DMEM) plus 1% sodium pyruvate, for HepG2, Hep3B, and HeLa, respectively. All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS). Incubation times, octreotide, and TNFa concentrations were chosen according to the results of our previous experiments [36–38].

2.3. Protocol for the Assessment of TNFRs

Cells seeded in 6-well plates were grown until confluent for the assessment of mRNA and the protein expression of TNFRs. Media were replaced twelve hours before stimulation with new medium without FBS. Growth-arrested cells were subsequently stimulated with octreotide 10⁻⁸ M, a clinically obtainable concentration, for the time periods indicated in Figures 1 and 2.

2.4. Reverse Transcription-PCR

RT-PCR was completed as previously described [36]. Trizol was used for the extraction of total RNA and treated with DNAse, according to the manufacturer’s instructions. Table 1 shows the primer pairs and the product size.

To ensure that primers for TNFR2 were functional, human genomic DNA was amplified in parallel, under identical PCR conditions. Resolution of PCR products was conducted by electrophoresis on 2% agarose gels. Staining with ethidium bromide allowed for the visualization of products. To check for genomic contamination, a similar parallel PCR (RT-negative PCR) accompanied each sample containing material without prior reverse transcription. Quantification of band densities was obtained by digital image analysis. The
density of each gene divided by that of 18S was used to quantify the results. Values were presented as fold difference compared to the untreated controls.

Table 1. Primer sequences used for the RT-PCR studies.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Product Size</th>
</tr>
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<tbody>
<tr>
<td>TNFR1</td>
<td>Sense: 5′-TCCTTCACCGCTTCAGAAAA-3′&lt;br&gt;Antisense: 5′-GGGATAGGCAAGGCAA-3′</td>
<td>418 bp</td>
</tr>
<tr>
<td>TNFR2</td>
<td>Sense: 5′-TGAAAGACCTGCACCTGGAAGA-3′&lt;br&gt;Antisense: 5′-GGGCTTGACACCACGTATTT-3′</td>
<td>176 bp (mRNA transcript) 683 bp (genomic DNA)</td>
</tr>
<tr>
<td>18s</td>
<td>Sense: 5′-AAACGGCTACACATCAAAC-3′&lt;br&gt;Antisense: 5′-CCTCAATGGATCCTCGTTA-3′</td>
<td>155 bp</td>
</tr>
</tbody>
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2.5. Western Blotting

Cells cultured with octreotide for 12 h were washed twice with PBS and treated with iced-cold lysis buffer (Distilled water with 50 mM Tris-HCL Ph 8.0, 4% CHAPS, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, and a mixture of protease inhibitors purchased from Roche, Basel, Switzerland) for 30 min. After centrifugation to discard insoluble debris (5 min, 10,000 × g, 4 °C), the protein concentration was measured (Bradford reagent, BioRad Laboratories, Hercules, CA, USA) with bovine serum albumin as the standard. Blotting was performed as previously described in detail [39]. Briefly, samples were mixed with one-third volume of 4× reducing Laemmli buffer and heated at 95 °C for 5 min. HepG2, Hep3B, and HeLa cell lysates (40 µg of protein) were put in each lane of the SDS-polyacrylamide gel electrophoresis and pre-stained protein molecular weight standards were also used. Proteins were subsequently transferred to polyvinylidene difluoride membranes and blocked for 1 h at room temperature in TBS supplemented with 0.05% Tween 20 and 5% skim milk. After an overnight incubation at 4 °C with rabbit anti-TNFR1 polyclonal antibody (1/200), membranes were re-incubated for 1 h at room temperature with a horseradish peroxidase-conjugated antibody (1/5000) and reactive bands were identified by chemiluminescence. Membranes were also stripped with a solution of 0.2 M NaOH and probed again with the anti-β-actin antibody (1/1000) to assess the equivalent loading and transfer.

2.6. NF-κB Localization by Immunofluorescence

HepG2 and Hep3B cells were cultured until 70% confluent and then serum-starved overnight. Quiescent cells were then pre-incubated for 12 h with or without octreotide 10⁻⁸ M in fresh medium without FBS, followed by treatment with octreotide 10⁻⁸ M, TNFa 30 ng/mL, and a mixture of octreotide–TNFa at similar concentrations for 30 min. NF-κB subcellular localization was examined using the NF-κB Activation HitKit (Thermo Fisher Scientific-Cellomics, Pittsburgh, Pennsylvania, PA, USA). After fixation in 3.7% formaldehyde solution, the cells were processed for immunofluorescence labelling following the manufacturer’s instructions. NF-kB was detected with an Alexa Fluor 488-conjugated secondary antibody and nuclei were labeled with Hoechst dye. In the end, slides were mounted in anti-fade mounting medium (Vector Laboratories, Burlingame, California, CA, USA) and visualized by standard fluorescence microscopy.

2.7. ELISA Determination of Total and Phosphorylated p65 in Intact Cells

HepG2 and Hep3B cells were seeded in 96-well plates at concentrations of 15 and 8 × 10³ cells/well, respectively, and after 24 h of culture, they were serum-starved overnight. Quiescent cells were then pretreated for 12 h with or without octreotide 10⁻⁸ M in fresh medium without FBS, followed by a further incubation with octreotide 10⁻⁸ M, TNFa 30 ng/mL, or a mixture of octreotide–TNFa for 15 min. After fixation in 4% formaldehyde solution, cellular levels of the total p65 and phosphorylated p65 (at serine 536 and serine 468) were measured using ELISA kits (NFkB p65 S536 CASE Kit, SA Biosciences Corporation, Frederick, Maryland, USA and NFkβ Profiler Kit, Active Motif Europe, Rixenhart, Belgium).
following the manufacturer’s recommendations. Immunoreactivity readings (OD450) were then normalized to cell numbers by measuring the protein concentration of each well (OD595). Results were presented as fold difference relative to the untreated controls.

2.8. Apoptosis Assays

**DNA fragmentation:** For the detection of apoptosis, the Cell Death Detection ELISA Plus Kit (Roche Diagnostics, Mannheim, Germany) was used. The assay specifically measures the histone-complexed DNA fragments from the cytoplasm of cells, after the induction of apoptosis. After 48 h of culture, the initial culture medium was substituted with media without serum, containing either octreotide (10^{-8} M), TNFa (30 ng/mL) or a mixture of the two for a further 24 h. Cell lysates were then used in ELISA, according to the manufacturer’s protocol. Briefly, after incubation, the cells were pelleted by centrifugation (200 × g, 10 min), and the supernatants were discarded. Cells were resuspended and incubated for 30 min in lysis buffer. After lysis, intact nuclei were pelleted by centrifugation. Aliquots of the supernatants were transferred to a streptavidin-coated well of a microtiter plate with two monoclonal antibodies, anti-histone (biotin labeled) and anti-DNA (peroxidase-conjugated). All samples were then incubated with the peroxidase substrate and absorbance was measured at 405 nm. The mean of the optical densities of eight untreated different controls was considered to be 100%, and all other values were expressed as fold changes of apoptosis [6,37,39].

**TUNEL assay:** In situ cell death (apoptosis) was further evaluated by terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick-end labelling (TUNEL) immunohistochemical staining. HepG2 and Hep3B cells were cultured in chamber slides for 48 h, followed by treatment with either octreotide 10^{-8} M, TNFa 30 ng/mL, or a combination of octreotide and TNFa for 12 h in serum free media. Cells were then fixed in 3.7% formaldehyde solution in PBS, subjected to the TUNEL assay using commercially available kits according to the manufacturers’ instructions, and visualized by light microscopy. TUNEL on HepG2 was performed with the TACS™ 2 TdT-DAB In Situ Apoptosis Detection Kit (Trevigen, Gaithersburg, MD, USA), resulting in a dark brown staining of the nuclei of apoptotic cells. TUNEL on Hep3B was performed with the In Situ Cell Death Detection AP (alkaline phosphatase) Kit (Roche, Basel, Switzerland). Fast Red was used as the substrate for the alkaline phosphatase reaction, yielding a red color on apoptotic nuclei. The slides were counterstained with Meyer’s hematoxylin. A TUNEL index was calculated by counting the total cells and the TUNEL positive cells of five visual fields (200 ×) in each section using the following formula: (number of stained cells/number of stained cells + number of unstained cells) × 100 [40].

2.9. Statistical Analysis

Results are presented as mean ± s.e.m. Between-group comparisons were made by one-way analysis of variance (one-way ANOVA) and the Bonferroni post hoc test for parametric values, or by a Kruskal-Wallis test and Dunn’s post hoc test for non-parametric values. Individual comparisons were made by the unpaired two-tailed Student’s t-test and p values < 0.05 were considered statistically significant. SPSS package version 15.0 for Windows (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA) were used for the statistical calculations.

3. Results

3.1. Modulation of TNFRs Expression in Hepatocellular Carcinoma Cell Lines by Octreotide

TNFR1 was identified to be present in basal conditions in both the HepG2 and Hep3B cells. In the HepG2 cells, octreotide reduced TNFR1 expression as early as after 1 h with a gradual restoration to normal values. In contrast, TNFR1 expression in Hep3B was later decreased at 6 and 12 h of octreotide stimulation (Figures 1A and 2A). Neither HepG2 nor Hep3B cells were found to express TNFR2 (Figures 1B and 2B).
Figure 1. Octreotide influence on TNFRs in HepG2 cells. (A) The basal expression of TNFR1 mRNA was reduced in the octreotide treated cells. Upper panel: RT-PCR of TNFR1 and 18s mRNA. Lower panel: Densitometry measurements of TNFR1 bands divided by those of 18s. Results are presented as fold difference in TNFR1 mRNA expression compared to the untreated control. One representative experiment similar to three independently performed experiments is shown. (B) TNFR2 was not expressed in the control or octreotide-treated cells, as confirmed by RT-PCR. Human genomic DNA was the positive control in the PCR reaction. (C) Western blot: Octreotide reduced protein TNFR1 expression. HeLa cells were the positive control (*p < 0.05, **p < 0.01 compared to controls).

Figure 2. Octreotide influence on TNFRs in Hep3B cells. (A) The mRNA TNFR1 basal expression was reduced after 6 h of treatment in cells stimulated with octreotide. Upper panel: RT-PCR of TNFR1 and 18s mRNA. Lower panel: Densitometry measurements of TNFR1 bands divided by those of 18s. Results are presented as fold difference in TNFR1 mRNA expression compared to the untreated control. One representative experiment similar to three independently performed experiments is shown. (B) TNFR2 was not expressed in the control or octreotide. Human genomic DNA was the positive control in the PCR reaction. (C) A slight decrease in TNFR1 55 kDa protein expression was detected (b-actin protein higher in octreotide lane). Note the two bands at 90 and 120 kDa. HeLa cells were the positive control (*p < 0.05 compared to controls).
TNFR1 protein expression in both hepatocellular carcinoma cell lines was evaluated by Western blotting. Prolonged overnight 12 h exposure of the blots showed a weak 55 kDa signal in the HepG2 lysates. Three faint bands of 55, 90, and 120 kDa in the Hep3B lysates were identified. TNFR1 protein levels at 55 kDa were clearly reduced in the HepG2 cells and were slightly decreased by octreotide in the Hep3B cells (Figures 1C and 2C).

3.2. Effect of Octreotide on Nuclear Translocation of NF-kB Induced by TNFa

In the resting HepG2 and Hep3B cells (Figures 3A and 4A) and octreotide-treated cells at a concentration of $10^{-8}$ M for 12 h (Figures 3B and 4B), cytoplasmically located NF-kB immunofluorescence was found. After the stimulation of quiescent cells with TNFa 30 ng/mL for 30 min, NF-kB accumulated in the nuclei, an effect most prominent in Hep3B (Figures 3C and 4C), whereas pre-incubation with octreotide $10^{-8}$ M for 12 h did not seem to inhibit the TNFa-induced nuclear translocation of NF-kB (Figures 3D and 4D).

3.3. Effect of Octreotide on Total and Phosphorylated p65 Levels

The addition of TNFa (30 ng/mL for 30 min) increased p65 phosphorylation at both serine 536 and 468 in the resting HepG2 and Hep3B cells and octreotide treated cells at a concentration of $10^{-8}$ M for 12 h (Figure 5; Figure 6, upper panels). TNFa-induced phosphorylation of p65 at both serine 536 and serine 468 was more evident in Hep3B. Interestingly, in HepG2, octreotide significantly limited TNFa-induced activation of p65 by reducing phosphorylation at serine 536 but not at serine 468 (Figure 5, upper panel). In Hep3B, however, octreotide had no effect on TNFa-induced serine 536 or serine 468 phosphorylation (Figure 6, upper panel), but slightly increased serine 468 phosphorylation on the resting cells. No statistically significant differences were observed in the total p65 levels in both cell lines.

**Figure 3.** Immunofluorescence analysis of NF-kB localization in HepG2 cells. NF-kB was detected with an Alexa Fluor 488-conjugated antibody and nuclei were labeled with Hoechst dye. No significant influence of octreotide on TNFa-induced NF-kB nuclear translocation was observed. (A) Untreated cells. (B) Cells treated with octreotide. (C) Cells stimulated with TNFa. (D) Cells pre-incubated with octreotide and stimulated with a combination of octreotide and TNFa.
Figure 4. Immunofluorescence analysis of NF-κB localization in Hep3B cells. NF-κB was detected with an Alexa Fluor 488-conjugated antibody and nuclei were labeled with Hoechst dye. TNFα induced a strong NF-κB nuclear translocation that did not appear to be affected by octreotide. (A) Untreated cells. (B) Cells treated with octreotide. (C) Cells stimulated with TNFα. (D) Cells pre-incubated with octreotide and stimulated with a combination of octreotide and TNFα.

Figure 5. Effect of octreotide on the phosphorylated and total p65 levels in HepG2 cells as determined by ELISA. Upper panel: TNFα alone or in combination with octreotide induced the activation of p65, which was phosphorylated at serine 536 and serine 468. Octreotide significantly limited the TNFα-induced activation of p65 by reducing phosphorylation at serine 536 but not at serine 468. Lower panel: Octreotide did not have any significant effect on the total p65 levels. Presented results are the mean of four independent experiments (** p < 0.01 different from control values, * § p < 0.05 from TNFα treatment).
of p65, which was strongly phosphorylated at serine 536 and serine 468. Octreotide itself caused a significant increase in P65 Ser468 phosphorylation and did not inhibit the TNFa-induced activation of p65. Lower panel: Octreotide did not have any significant effect on the total p65 levels either alone or in combination with TNFa. Presented results are the mean of three independent experiments (* p < 0.05, ** p < 0.01 different from control values).

3.4. Differential Apoptotic Response of Human Hepatoma Cell Lines to TNFa and Octreotide

Octreotide or TNFa alone induced a non-significant apoptosis in HepG2, while their combined treatment resulted in significantly increased apoptotic cell death (Figure 7). DNA fragmentation analysis in Hep3B (Figure 8) revealed significantly elevated levels of nucleosomes after incubation with TNFa. Octreotide alone did not have any apoptotic effect and significantly reduced TNFa-induced apoptosis.

![Figure 6](image1.png)

**Figure 6.** Effect of octreotide on the phosphorylated and total p65 levels in Hep3B cells as determined by ELISA. Upper panel: TNFa alone or in combination with octreotide induced the activation of p65, which was strongly phosphorylated at serine 536 and serine 468. Octreotide itself caused a significant increase in P65 Ser 468 phosphorylation and did not inhibit the TNFa-induced activation of p65. Lower panel: Octreotide did not have any significant effect on the total p65 levels either alone or in combination with TNFa. Presented results are the mean of three independent experiments (* p < 0.05, ** p < 0.01 different from control values).

![Figure 7](image2.png)

**Figure 7.** Octreotide or TNFa alone did not cause any significant apoptosis in HepG2 cells as detected by the DNA fragmentation method, whereas their combination resulted in statistically significant apoptotic cell death (* p < 0.05 compared to control values).
A 12 h exposure to TNFα 30 ng/mL resulted in significant numbers of apoptotic Hep3B cells (TUNEL index 43 ± 7, Figure 9A, Figure 10 compared to 19 ± 5 for HepG2 p < 0.05). Octreotide 10^{-8} M alone induced slight apoptosis after 12 h of treatment, but when combined with TNFα, it decreased the population of TUNEL-positive nuclei (25 ± 3, p < 0.05, Figures 9B and 10).

Figure 8. TNFα significantly increased apoptotic death in Hep3B cells whereas octreotide reduced cell sensitivity to TNFα-mediated apoptosis. Hep3B was incubated with either octreotide, TNFα, or their combination (* p < 0.05 compared to control values, § p < 0.05 compared to TNFα treatment).

Apoptotic DNA fragmentation was further verified using TUNEL assays. A 12 h exposure to TNFα 30 ng/mL resulted in significant numbers of apoptotic Hep3B cells (TUNEL index 43 ± 7 Figure 9A, Figure 10 compared to 19 ± 5 for HepG2 p < 0.05). Octreotide 10^{-8} M alone induced slight apoptosis after 12 h of treatment, but when combined with TNFα, it decreased the population of TUNEL-positive nuclei (25 ± 3, p < 0.05, Figures 9B and 10).

Figure 9. A representative picture of the TUNEL assay. The effect of TNFα alone or in combination with octreotide on apoptosis detected by the TUNEL assay in Hep3B cells. (A) TNFα induction of apoptosis. (B) TNFα plus octreotide reduced apoptotic cells. Original magnification ×200.

After 12 h of treatment, few apoptotic nuclei were detected in the HepG2 cells incubated with either serum free medium (control cells), octreotide 10^{-8} M, or TNFα 30 ng/mL, whereas the combination of octreotide and TNFα clearly increased the TUNEL-positive cells (38 ± 4 p < 0.05, Figure 10).
After 12 h of treatment, few apoptotic nuclei were detected in the HepG2 cells incubated with either serum free medium (control cells), octreotide $10^{-8}$ M, or TNFa 30 ng/mL, whereas the combination of octreotide and TNFa clearly increased the TUNEL-positive cells ($38 \pm 4$, $p < 0.05$, Figure 10).

**Figure 9.** A representative picture of the TUNEL assay. The effect of TNFa alone or in combination with octreotide on apoptosis detected by the TUNEL assay in Hep3B cells. (A) TNFa induction of apoptosis. (B) TNFa plus octreotide reduced apoptotic cells. Original magnification $\times 200$.

**Figure 10.** TUNEL index of HepG2 and Hep3B demonstrating the difference in apoptosis index after co-incubation with octreotide and TNFa in the two cell lines (* $p < 0.05$ compared to control values, § $p < 0.05$ compared to TNFa treatment).

### 4. Discussion

Somatostatin is a hormone involved in various cellular functions including proliferation, differentiation, and apoptosis. Many studies have reported that somatostatin may induce cell death in normal and carcinoma cells [41–45]. Moreover, somatostatin mediates apoptosis in many cancerous cell lines [46–48]. Clinical studies using the synthetic somatostatin analog octreotide as a monotherapy of inoperable HCC have produced conflicting results [8]. In this study, we examined the hypothesis that conflicting results may be partially due to the fact that octreotide may have different results in different cell lines.

Both ourselves and other authors [6,49] have previously demonstrated that octreotide and TNFa increase apoptosis in HepG2 cells. Therefore, we studied some aspects of apoptosis in two liver carcinoma cell lines. HEPG2 is a hepatoblastoma cell line that was isolated from the liver biopsy of a young Caucasian male [50]. These cells produce various plasma proteins like albumin, alpha2-macroglobulin, alpha 1-antitrypsin, and transferrin but they do not express hepatitis B virus surface antigens. On the other hand, Hep3B cells isolated from an 8-year-old Colored male integrated the hepatitis B virus genome but did not produce infectious hepatitis B virus particles [51,52].

In our study, we found that TNFR1 could be identified in the two cell lines before any stimulation while TNFR2 was not constitutively expressed. Octreotide caused an early (within the hour) decrease in both the mRNA and protein of TNFR1 in HepG2 compared to a later reduction at 6 and 12 h in the Hep3B cells. The formation of homodimers or homotrimers by TNFRs in cell surfaces [53] could provide an explanation for the vague bands of 90 kDa observed in the TNFR1 blots of Hep3B lysates, while the bands of approximately 120 kDa on the same blots may represent TNFR1-TNFa heterocomplexes, but this requires further research. This octreotide effect on TNFR1 contrasts previous results on other cell lines. In a recent paper using NIH3T3 cells, somatostatin receptor subtype 2 (sst2) activation increased the expression of the TNFR1 protein and subsequently induced the
cleavage of procaspase-8, leading to the upregulation of apoptosis caused by stimulation with TNFa [20]. Increased expression of TNFR1 mRNA caused by IL-6 also led to the upregulation of TNFa-induced apoptosis in rat hepatocytes [54]. In contrast, sst2 activation had no effect on TNFR1 mRNA expression in a pancreatic cancer cell line, but it caused an increase in TNFR1 protein, indicating that sst2 activation affects the post-transcriptional level [48].

Octreotide had no effect on either NF-kb translocation or apoptosis as detected by the DNA fragmentation assay or the TUNEL assay in both cell lines. This may be due to the methods used to detect apoptosis. The degradation of DNA as detected by the DNA fragmentation assay is a characteristic of apoptosis, but necrosis can also produce similar DNA cleavage. Therefore, an additional method should confirm apoptosis. The TUNEL method identifies cells with DNA strand breaks in situ by using terminal deoxynucleotidyl transferase (TdT) to transfer biotin-dUTP to the cleaved ends of the DNA. Although TUNEL may also be positive in necrosis, the two methods combined do reflect apoptosis [55–57].

In fact, in our previous study in HepG2 cells, DNA fragmentation also failed to detect apoptosis changes after octreotide or TNFa incubation. Only Annexin B detected these changes, which were confirmed by caspase 8 measurements [6]. The TNFR1 receptor is activated after TNFa binding, which results in NF-kb nuclear translocation [58]. This was indeed our finding in both cell lines, with a higher translocation in the Hep3B cells. However, contrary to expectation, translocation was associated with increased apoptosis in these cells.

This finding poses the question of the role of NF-kb in apoptosis in liver cells. Is NF-kb activation an anti-or a pro-apoptotic factor for hepatocytes? NF-κB activation in the liver parenchymal cells depends on TNFa production by Kupffer cells. The inhibition of TNFa signaling reduces the expression of NFκb dependent pro-survival genes, leading to increased hepatocyte apoptosis [25,59]. NF-kb has been considered an anti-apoptotic molecule [60]. Moreover, the inhibition of NF-kb by several agents increases apoptosis, mostly in colon cancer cells [61–64]. A recently described mechanism is that this is achieved through the action of serine/threonine kinase glycogen synthase kinase-3β (GSK-3β), which inhibits apoptosis through the activation of NF-κB [65]. However, reports have been published indicating that NF-kb activation may in fact induce apoptosis in various cells including fibroblasts [66,67], T cells [68,69], osteoblasts [70], and neurons from Parkinson’s disease patients [71]. More recently, the Src-associated substrate during mitosis of 68 KDa (Sam68) increased apoptosis in intestinal epithelial cells and articular chondrocytes by activating NF-kb [72,73]. Our findings also showed a dual effect of TNFa on p65 serine 536 and serine 468 phosphorylation, and ultimately, apoptosis was identified by both DNA fragmentation and the TUNEL assay. In the Hep3B cells, p65 phosphorylation and apoptosis were more pronounced compared to the HepG2 cells. Such a dual effect of TNFa and NF-kb activation on apoptosis has been reported in an earlier report where TNFx activation of TNFR1 may lead to either cell survival or cell death [74]. Most interestingly, it was shown in intestinal epithelial cells that transient NF-kb activation led to decreased apoptosis, while chronic NF-kb activation led to enhanced apoptosis [75–77]. This is due to the effect of the receptor interacting protein kinase 1 (RIPK1). Association of this kinase with the adaptor protein TNFR1-associated death domain protein (TRADD) after TNFR1 activation by TNFa will lead to the expression of anti-apoptotic molecules such as Bcl-2 and the inhibition of apoptosis. Dissociation of this complex leads to the association of RIPK1 with the adaptor FAS-associated death domain protein (FADD), which will activate caspase 8 and induce apoptosis. The different temporal reduction in TNFR1 in the HepG2 and Hep3B cells in our study might therefore be an explanation for the different apoptotic response of the two cell lines [78,79]. In addition, the different results of the TNFa effect on NF-kb translocation and apoptosis might be explained by the finding that a transient activation of c-Jun N-terminal kinase (JNK) by TNFa favors cellular survival while a protracted activation, as in our study, leads to apoptosis [80]. In this respect, somatostatin has been reported to increase apoptosis through the inhibition of anti-apoptotic JNK, mediated by the sst2 receptor [20,81,82].
The measurement of p65 activity is considered to represent NF-κB activity, which mostly reflects the canonical pathway [17]. p65 is used to demonstrate the first cytosolic step of NF-kB activation. The downregulation of p65 inhibited inflammatory responses and hepatocarcinogenesis [83,84]. In our study, the total p65 was not different between the two cell lines. However, it is the phosphorylation of NF-kB in several sites that drives hepatocarcinogenesis through the inhibition of apoptosis [29]. In our study, the phosphorylation of p65 was increased, mostly in serine 468 of the Hep3B cells. However, at least in Hep3B, the increase in p65 serine phosphorylation increased apoptosis. In fact, this is in agreement with earlier studies reporting that stimulation with TNFa of an HBV hepatoma cell line induced apoptosis mediated by HBV protein X. As previously mentioned, Hep3B cells express HBV [85,86]. However, a different explanation of our findings is plausible. The p65-deficient cells where the p65 protein was substituted with a p65 protein mutated for serine 468 suggest a negative regulatory role of serine 468 for NF-κB activation. Increased phosphorylation of Ser 468 led to a reduction in NF-kB activation, hence increased apoptosis. This was indeed the case in the Hep3B cells compared to HepG2 [87,88].

Co-incubation of cells with TNFa and octreotide had different effects in NF-kB phosphorylation. Although octreotide by itself had no effect in P65 Ser536 phosphorylation as previously mentioned, it caused a reduction in TNFa-induced phosphorylation only in the HepG2 cells. TNFa also increased P65 Ser468 phosphorylation more evident in Hep3B, and this effect was not affected by co-incubation with octreotide in both HepG2 and Hep3B cell lines. However, co-incubation with octreotide and TNFa produced completely different results in the two cell lines. Apoptosis was significantly increased in the HepG2 cells. In contrast, the Hep3B cells, which were very sensitive to TNFa-induced apoptosis, had significantly reduced apoptosis after co-incubation. Importantly, these differences could not be explained by different p65 phosphorylation, since no effect on phosphorylation was observed after co-incubation. It is possible, however, that this difference might be due to the 12 h period of co-incubation, as by that time, the reduction in TNFR1 expression was restored in HepG2, but not in the Hep3B cells. Our results may partially explain the conflicting results of clinical trials and experimental evidence [8,89]. Thus, octreotide has been shown to induce apoptosis in prostate cells or Kupffer cells [39,90]. It has also shown to inhibit apoptosis in Kupffer cells after prolonged incubation [91] and rabbit hepatocytes [92] or liver and kidney cells after hepatic-reperfusion injury [40,93]. Explanations of the effect on apoptosis by the combination of octreotide and TNFa are further complicated, since there is evidence that TNFa itself decreases the expression of somatostatin receptors [94].

5. Conclusions

The purpose of this study was to support the hypothesis that octreotide might have different effects on apoptosis and the TNFa pathway in different hepatocellular cell lines. Our results clearly support this hypothesis. Therefore, these findings might in part explain the discrepancies in response to octreotide treatment in different trials of HCC patients. In light of our results on the effect of octreotide on TNFR1 expression, it seems plausible that octreotide does not have a major direct effect on apoptosis as it does not affect either NF-kB nuclear translocation or P65 Ser536 phosphorylation. Five explanations are proposed for the different results in the two cell lines tested. (1) Prolonged incubation as opposed to shorter incubation periods. (2) Differential phosphorylation of Ser 536 and Ser 468. (3) The actual expression of TNFR1 is not the only factor regulating TNFa-induced apoptosis. Glycation and sialylation of the receptors are additional factors not tested in the present study. (4) Different interactions of NF-kB with p53 in the two cell lines. (5) TNFa stimulates both NF-kB and JNK activities, which have opposite actions on cell survival. Further research is clearly indicated to test in detail these possible mechanisms as their clarification will allow for the better selection of patients suitable for optimal treatment results and possible combinations with anti-TNFa agents.

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I.T. and A.V.: Assessed the results and the initial draft and searched the literature. I.D. and G.E.: Performed part of the laboratory work. E.K.: Devised the study, supervised the project, and finalized the manuscript. All authors have read and agreed to the published version of the manuscript.

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**Informed Consent Statement:** Not applicable. No human subjects were involved.

**Data Availability Statement:** The data collected and/or analyzed during this study are available from the corresponding author on reasonable request.

**Conflicts of Interest:** The authors declare that they have no conflicts of interest.

**Abbreviations**

- AP: alkaline phosphatase
- DAB: 3,3′-diaminobenzidine
- DMEM: Dulbecco’s modified Eagle’s medium
- dNTPs: deoxyribonucleotide triphosphates
- FBS: fetal bovine serum
- HCC: hepatocellular carcinoma
- HRP: horseradish peroxidase
- IkB: inhibitory kB
- IKK: IkB-inducing kinase
- IL: interleukin
- JNK: c-Jun N-terminal kinase
- kDa: kilodaltons
- NF-kB: nuclear factor-kappa B
- RT: reverse transcription
- Sst2: somatostatin receptor subtype 2
- TBS: Tris-buffered saline
- TDT: terminal deoxyribonucleotide transferase
- TNFR1: TNF receptor 1
- TNFR2: TNF receptor 2
- TNFRs: tumor necrosis factor receptors
- TUNEL: terminal deoxyribonucleotide transferase-mediated dUTP nick-end labelling

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