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Histone deacetylases affect transcriptional regulation of CCL2 and CXCL8 expression by pulmonary fibroblasts *in vitro*

Epigenetic regulation of CCL2 and CXCL8 expression

Abstract

Introduction: Chemokines have been shown to play an important role in tissue remodeling and fibrosis in the respiratory system. In this study we wanted to evaluate the mechanisms, which regulate the expression of selected chemokines by pulmonary fibroblasts *in vitro*.
Material and methods: Pulmonary fibroblasts were cultured with and without bacterial lipopolysaccharide (LPS) for 6 hours. In addition some of the cultures were pre-treated with histone deacetylase inhibitor Trichostatin A (TSA). Real-time PCR reaction was performed to estimate the expression of chemokines CCL2, CCL3 and CXCL8.
Results: In unstimulated cultures detectable expression of CCL2 and CXCL8 was observed, while CCL3 expression could not be detected. After stimulation with LPS, TSA and both agents together CCL2 expression rose by 1.52, 1.62 and 1.8 times in comparison to control cultures respectively. CXCL8 mRNA expression levels after stimulation with LPS, TSA and LPSTSA increased by 1.53, 1.91 and 2.4 times accordingly.
Conclusion: Epigenetic mechanisms related to histone acetylation affects transcriptional regulation of CCL2 and CXCL8 expression by pulmonary fibroblasts. Those mechanisms may play a role in tissue repair and pathologic remodeling.

Key words: CCL2, CXCL8, histone deacetylase inhibitors, fibroblasts, real-time polymerase chain reaction

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Introduction

Fibroblasts are involved in tissue repair after injury, but in chronic pathologic conditions they are responsible for remodeling such as excessive fibrosis, which leads to irreversible impairment of organ functions [1]. It is particularly important in the respiratory system where fibrotic processes participate in obstructive and restrictive lung diseases [2]. Both conditions lead to lung function impairment and finally respiratory failure and death. In fact, chronic obstructive pulmonary disease and pulmonary fibrosis are leading causes of morbidity and mortality due to chronic respiratory diseases [3]. Fibroblasts themselves actively participate in regulation of inflammatory response and tissue repair by secretion of a broad array of biologically active mediators such as cytokines and chemokines [4].

Fibroblasts represent a heterogeneous population of cells, which differ in their pro-fibrotic potential and the profile of released mediators [5]. Residing in different organs fibroblasts adjust their phenotype and function [6]. Several studies demonstrated that fibroblasts from different organs may differ in their ability to produce biologically active mediators [7–9]. Similarly, different pathologic conditions may lead to changes in fibroblast phenotype, the most pronounced being the transformation of fibroblasts into myofibro-

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blasts seen in chronic inflammatory diseases such as asthma [10]. The altered fibroblast phenotypes are manifested not only *in vivo* but also *ex vivo*, as demonstrated in cell culture experiments [11]. The persistence of altered fibroblast phenotype *ex vivo* indicates that some genetic and/or epigenetic mechanisms may be involved in this process. The mechanisms leading to altered fibroblast phenotype may affect the profile of biologically active mediators released by individual cells.

Among chemokines produced by fibroblasts and involved in the process of tissue remodeling and fibrosis CCL2 and CXCL8 are of particular interest. It has been already demonstrated that CCL2 plays a crucial role in the process of tissue fibrosis in chronic diseases which are characterized by excessive fibrosis, such as systemic sclerosis [12]. In patients with systemic sclerosis CCL2 is overexpressed by fibroblasts derived from the skin [12]. Moreover, *ex vivo* elevated production of CCL2 by peripheral blood mononuclear cells derived from systemic sclerosis patients has also been demonstrated [13]. In addition exacerbation of chronic diseases such as allergic asthma is associated with increase of plasma CCL2 concentration [14].

Fibroblasts are also a major source of CXCL8 in chronic pulmonary diseases [15]. Fibroblasts derived from the airways of chronic obstructive airway disease (COPD) patients release more CXCL8 upon stimulation with WNT-5 ligands than those from healthy subjects [16]. Elevated levels of CXCL8 are produced by fibroblasts derived from tissues undergoing pathological remodeling. In particular, fibroblasts from nasal polyps release more CXCL8 than those from nasal mucosa derived from patents without nasal polyps [17]. Moreover, human lung fibroblasts release CXCL8 and CCL2 upon stimulation with Mycoplasma antigens indicating that those chemokines are involved in innate immune response [18].

Regulation of the CCL2 expression by different cells has been demonstrated on genetic and epigenetic levels [19–22]. Association of genetic polymorphisms which alter CCL2 expression with different pathological processes have been already demonstrated [19, 20]. Among genetic factors an A-2578G single nucleotide polymorphism, which is functional and affects the expression of CCL2 is associated with susceptibility to some chronic diseases such as asthma or lupus nephritis [19, 20]. Moreover, altered histone acetylation plays a role in the regulation of CCL2 expression indicating on an important role of epigenetic factors on CCL2 expression [21]. Similarly, functional polymorphisms of CXCL8 and its receptor has been shown to confer predisposition to systemic sclerosis [22].

The aim of this study was to evaluate mechanisms, which regulate the expression of chosen chemokines *in vitro* by pulmonary fibroblasts.

Material and methods

Cell culture: Human lung fibroblast cell line HLF 506-05a derived from normal human lung parenchyma was obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco Modified Eagle Medium (DMEM) with sodium pyruvate, L-glutamine and 3.7 g/L sodium bicarbonate (PAN-Biotech, Aidenbach, Germany) supplemented with 10% Fetal Bovine Serum (Sigma-Aldrich St Louis, MO, USA), Penicillin (100 U/ml), Streptomycin (0.1 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA), 5% MEM Non-Essential Amino Acids Solution (Gibco, ThermoFisher Scientific, Waltham, MA, USA) and 0.5% 2-mercaptoethanol (Sigma-Aldrich). In addition, obtained from ATCC, human gingival fibroblasts cell line HGF-1 was used as a comparator. Preliminary culture was carried out under normal air conditions in a 5% carbon dioxide atmosphere at 37°C in cell culture flasks for adherent cells. After incubation for 20 hours with or without Trichostatin A (Sigma-Aldrich) at a concentration of 250 nM the cells were stimulated with 2.5 mg/ml bacterial endotoxin (Sigma-Aldrich) for 6 hours. After the stimulation the cells were lysed in RA1 buffer, collected with the use of RNAlater (Sigma-Aldrich) and frozen in –80°C until tested.

RNA isolation: Isolation of RNA was performed on the frozen cells lysate with the use of the NucleoSpin® RNA II Kit according to manufacturer protocol. Quality, purity and concentration of isolated RNA was evaluated with spectrophotometric method on NanoDrop 2000.

Reverse transcription: Reverse transcription reaction was performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer protocol. The PCR amplification was carried out in 10 μL reaction mix consisting of MultiScribe™ Reverse Transcriptase 0.5 μL, 25 × dNTP Mix (100 mM) 0.4 μL, 200ng of tested RNA and 1 μL of both primers and RT buffer supplied by the manufacturer. Reactions were performed with the use of recommended by the manufacturer thermal cycling conditions.

Gene expression estimation: To estimate expression profiling for selected genes, real-time

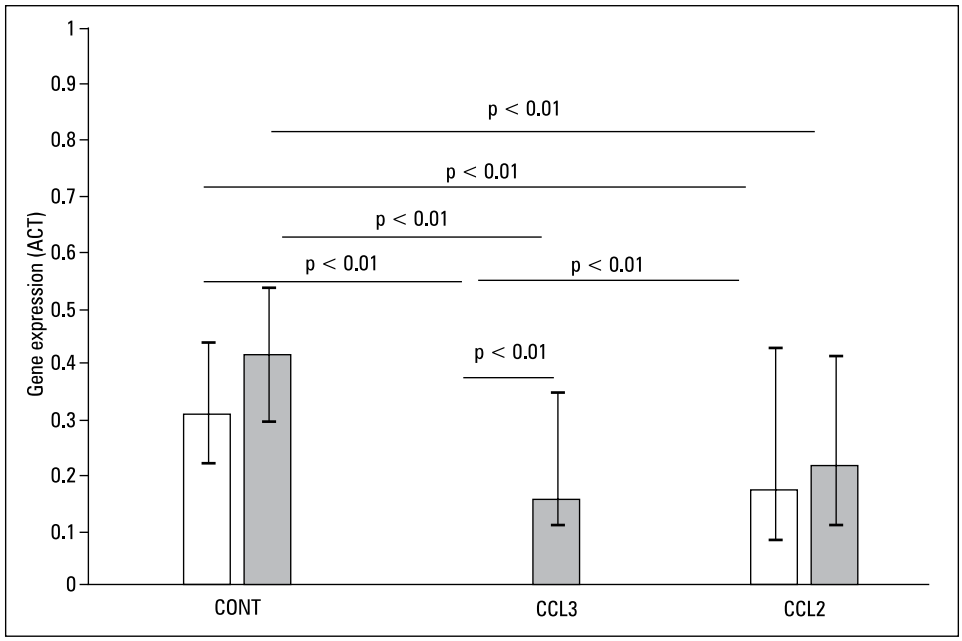

Figure 1. mRNA expression of chosen cytokines in unstimulated cultures of pulmonary (open bars) and gingival (gray bars) fibroblasts

RT-PCR analysis was performed as described previously. We report data for gene-expression TaqMan assays for CCL2 (NM_002982), CCL3 (NM_002983) and CXCL8 (NM_000584.3). The relative expression of target mRNA was normalized to the housekeeping gene of β-2-microglobulin (NM_002982.3).

Quantitative estimation of gene expression: To estimate the relative expression level (R) of tested genes we used $\Delta\Delta C_T$ comparative analysis. As an endogenous and exogenous control was used expression level of β-2-microglobulin gene and expression levels of unstimulated samples accordingly. In the first step we calculated ΔC_T between tested genes and endogenous control. Next we computed $\Delta\Delta C_T$ between stimulated and unstimulated samples and calculated relative expression levels between both samples.

Statistical analysis: Statistical analysis and graphs were done in Medcalc. Results are presented as median ± interquartile ranges (IQR). MRNA expression levels of each chemokine were calculated as the percentage of endogenous control mRNA level. Results were obtained through calculation of ΔC_T. Statistical evaluation of obtained results was executed with the Kruskal-Wallis test. Evaluation of differences between tested cytokines was performed with the use of Wilcoxon test.

Results

In the unstimulated lung fibroblasts the greatest expression among the studied chemokines was detected for CXCL8 (ΔC_T = 0.313; 0.228–0.435), which was significantly greater than that of CCL2 (ΔC_T = 0.174; 0.084–0.435; $p < 0.05$) (Fig. 1). Comparing the numerical values of basal mRNA expression the expression of CXCL8 was almost twice as high as the expression of CCL2. In vitro expression of CCL3 in pulmonary fibrobasts was not detected after 40 cycles of PCR amplification. In contrast to the lung fibroblasts, significant expression of CCL3 (ΔC_T = 0.16; 0.07–0.35) was detected in the gingival fibroblasts which was comparable to that of CCL2 (ΔC_T = 0.22; 0.12–0.35; $p = 0.2$) and less than that of CXCL8 (ΔC_T = 0.425; 0.3–0.54; $p < 0.01$).

In order to evaluate potential effect of activation status on differences in individual chemokine expression between lung and gingival fibroblasts the former cells were stimulated with LPS. Moreover, to evaluate a potential effect of histone acetylation on specific pattern of chemokine expression by lung fibroblasts was evaluated.

Stimulation with LPS resulted in significant up-regulation of CCL2 and CXCL8 and no effect on CCL3 expression by the lung fibroblasts.

In vitro expression level of CCL2 mRNA in stimulated by LPS pulmonary fibroblast cultures was 1,52 fold greater than in the control cultures (1.122–1.838; $p < 0.05$) and it was similar to that in cultures stimulated by TSA where CCL2 expression was 1,62 fold greater than in the control cultures (1,5–1,805; $p = 0.4$) (Fig. 2). Stimulation of TSA pre-treated lung fibroblasts with LPS resulted in 1.8 fold (1.55–2.1) increase of

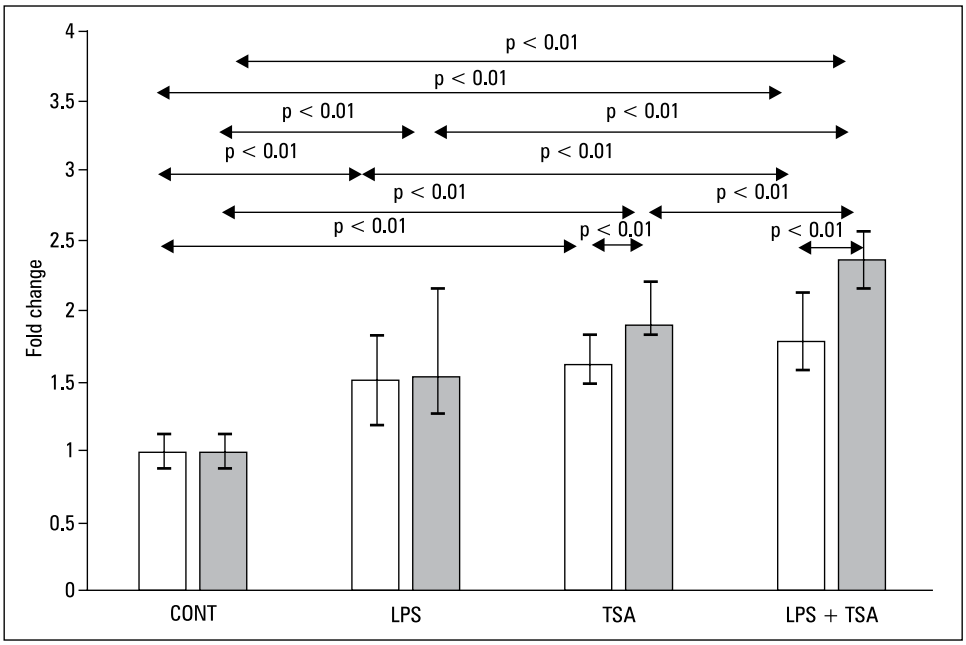

Figure 2. Changes of CCL2 (empty bars) and CXCL8 (gray bars) expression in response to LPS and/or TSA stimulation. Only significant differences are marked

CCL2 expression in comparison to unstimulated cultures. The effect was slightly greater than that of LPS alone ($p < 0.05$) but did not differ significantly from that seen in TSA stimulated cultures ($p = 0.1$).

Pulmonary fibroblasts stimulated with LPS were characterized by 1.53 fold (1.28–2.15) greater CXCL8 than in the control cultures ($p < 0.01$). In the cells treated with TSA the expression of CXCL8 was higher (1.91 fold; 1.78–2.16) and it was significantly greater that in the control cultures ($p < 0.01$) being also greater than in the cells stimulated with LPS alone ($p < 0.05$). Concomitant treatment of pulmonary fibroblasts with TSA and LPS resulted in 2.41 fold (2.1–2.53; $p < 0.01$) up-regulation of CXCL8 expression (Fig. 2) which indicates the additive effect of individual stimuli.

Discussion

Our study demonstrates *in vitro* differences in a pattern of CCL2, CXCL8 and CCL3 mRNA expression by fibroblasts derived from different anatomical localizations with a special attention to pulmonary fibroblasts. The results indicates on differences in transcriptional regulation of individual chemokines in relation to the site of origin of individual fibroblasts. Interestingly, we demonstrated the lack of CCL3 expression in the lung fibroblasts both spontaneously and after stimulation with endotoxin. However, CCL2 and CXCL8 were expressed and the level of their expression was significantly up-regulated after LPS stimulation. This is coherent with the study results of Daenga *et al.* [23] in which endogenous expression of CCL2 mRNA in pulmonary fibroblasts derived from healthy subjects was demonstrated and significantly higher expression of this chemokine mRNA levels in pulmonary fibroblasts of people with idiopathic pulmonary fibrosis was detected [23]. Similarly, another study demonstrated significant expression of CXCL8 by pulmonary fibroblasts [24]. Finally, the lack of basal expression of CCL3 mRNA in pulmonary fibroblasts is coherent with the study of Brouty-Boyé *et al.* [25] in which CCL3 expression by bone marrow and breast, but not pulmonary fibroblasts was detected [25]. All this studies provided evidence for functional heterogeneity of fibroblasts derived from the lungs and gingiva.

Since differences in CCL3 mRNA expression between pulmonary and other fibroblasts could potentially indicate differences in epigenetic control we decided to evaluate the effect of TSA on expression of the studied chemokines. Our study did not support the concept which assumed that histone acetylation played a major role in regulation of CCL3 expression. It seems that more stable mechanisms of CCL3 gene repression operate in pulmonary fibroblasts.

Moreover, we were able to demonstrate no additive effect of TSA on LPS-stimulated CCL2 expression which again argue against little role

of histone acetylation in the expression of that chemokine by pulmonary fibroblasts. Most of the CC chemokines, including CCL2 and CCL3 are clustered on a long arm of chromosome 17 (17q11.2–q21.1) which indicates that similar level of histone acetylation in that region may be seen. Interestingly, in other cell types additive effect of TSA on LPS-stimulated CCL2 expression was observed [26, 27]. The additive effects were demonstrated for murine preadipocytes and macrophages [26, 27]. On the other hand inhibitory effect on CCL2 expression was detected in aortic endothelial cells [28]. The results of the presented studies indicate that cells from various anatomical regions and tissues differently react to hiperacethylation of histone tails inducted by TSA and a small amount of studies typify this occurrence does not allow to place unequivocal conclusions.

In contrast to CC chemokines we were able to demonstrate a significant effect of TSA on expression of CXCL8 both spontaneously and after LPS stimulation. These results indicate an addictive influence of histone tail acetylation induced by TSA with LPS-induced cell activation on CXCL8 mRNA expression in pulmonary fibroblast *ex vivo*. Similar results were obtained by Iwata et al. who observed growth of CXCL8 expression in lung epithelial cells transformed with SV-40 stimulated by LPS and TSA in comparison to those cells stimulated by LPS alone [29]. Angrisano *et al.* [30] received also congruous results using human derived colon cells. Angrisano and coworkers observed increased expression of CXCL8 mRNA in cultures stimulated by LPS and TSA in comparison to stimulation with LPS alone and also noted differences in acetylation and methylation of histone tails in CXCL8 gene promoting region after stimulation with LPS [30]. Interestingly, CXCL8 is localized on the long arm of chromosome 4 (4q13.3) and therefore local differences in histone acetylation may explain the differences of TSA effect on CCL2 and CXCL8 expression.

Therefore, therapeutic agents which affect histone acetylation may exert different effect on the expression of individual chemokines in pulmonary fibroblasts. Also, the effect of those therapeutic agents may differ depending on the anatomical localization of fibroblasts. It should be emphasized however, that in this study we focused only on a one mechanism of epigenetic control of chemokine expression. It is likely that other epigenetic and genetic factors may affect expression of those chemokines in clinical practice. Further studies are warranted to solve the mechanisms responsible for regulation of fibroblast phenotypes. This may be particularly important in search for effective treatment of diseases which are characterized by pathological tissue remodeling with fibrosis such as pulmonary fibrosis and COPD.

Conclusions

In summary, our study demonstrates the complex regulation of chemokine expression by pulmonary fibroblasts. It indicates the differences between epigenetic regulation of CXCL8 and CCL2/CCL3 expression indicating on a possible role of pulmonary fibroblasts as a source of pro-inflammatory chemokines CCL2 and CXCL8 in the pathogenesis of chronic inflammatory lung diseases.

Conflict of interest

The authors declare no conflict of interest.

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