Pharmacological Reports
2010, 62, 1139–1147
ISSN 1734-1140

Lactoferrin inhibits the growth of nasal polyp fibroblasts

Beata Nadolska[1], Marcin Frączek[1], Tomasz Kręcicki[1], Maja Kocięba[2], Michał Zimecki[2]

[1]Department of Otolaryngology, Head & Neck Surgery, Wroclaw Medical University, Borowska 213, PL 50-556 Wrocław, Poland

[2]Institute of Immunology and Experimental Therapy, Weigla 12, PL 53-114 Wrocław, Poland

Correspondence: Michał Zimecki, e-mail: zimecki@iitd.pan.wroc.pl

Abstract:
The aim of this study was to evaluate the effects of lactoferrin (LF) on the growth of fibroblasts derived from nasal polyps. We showed that the proliferation of fibroblasts was inhibited in a dose-dependent manner by both native and recombinant LF. The greatest inhibition of proliferation was caused by human milk-derived, iron-saturated LF. The inhibition of fibroblast proliferation was not species specific because bovine LF also was active. The interaction between LFs and a putative cell receptor did not depend on the sugar composition of the glycan moiety of the LF molecule because lactoferrins of different origins were active and the addition of monosaccharides to the cultures did not block proliferation. However, the treatment of fibroblasts with sodium chlorate (an inhibitor of glycosaminoglycan sulfation) or the addition of heparin abolished the inhibitory effect of LF, suggesting that LF binds heparan sulfate-containing proteoglycans. The significance of LF in nasal excretions in controlling polyp formation is discussed.

Key words:
nasal polyps, fibroblasts, lactoferrin, heparan sulfate

Introduction

Nasal polyposis is an inflammatory disorder of the sinonasal mucosa of unknown etiology. Chronic inflammation causes a reactive hyperplasia of the intranasal mucosal membrane, which results in the formation of polyps. Nasal polyps (NP) consist of columnar ciliated and stratified squamous epithelium covering swollen connective tissue built with fibroblasts, dilated capillaries and venous channels [29]. Upon stimulation, fibroblasts, *via* production of collagens and fibronectin, promote extracellular matrix generation, tissue remodeling and, consequently, stromal and epithelial abnormalities [35]. NPs are characterized by intense tissue eosinophilia that is subsequently associated with a T-helper type 2 cytokine profile [3].

Only a small proportion of NPs can be connected with genetic disorders. In the majority of cases, the etiopathology is multifactorial, often related to both the host and the environment. The factors influencing NP development include altered local homeostasis of nasal mucosa, bacterial and fungal colonization, allergy, neurovascular factors and poorly understood disturbances of the innate defense system [5].

Mucosal secretions play a crucial role in nonspecific defense against pathogens [28]. Lactoferrin (LF) represents one of the major components of mammalian secretions and plays numerous roles in innate and adaptive immunity [46]. LF belongs to the family of proteins involved in iron metabolism. The protein exists in two separate reservoirs, in body secretions [24] and in neutrophils [15]. Receptors for LF can be found in nearly all organs and cell types [38]. LF can interact with, among others, major cell receptors associated with innate immunity such as the mannose receptor [45], CD14 [13], toll-like receptors [11], CD169 sialoadhesin [10] and heparan sulfate [25].

The predominant role of LF is associated with nonspecific defense against pathogens including bacteria [8], fungi [40], viruses [36] and parasites [20]. LF regulates myelopoiesis [1], promotes maturation of T [47] and B cells [48] and exhibits adjuvant activity [17, 45]. Its important roles in host defense include anti-inflammatory properties [4], in particular anti-oxidant actions [19]. LF was also found to inhibit mitogen-induced proliferation and the mixed lymphocyte reaction of human peripheral blood mononuclear cells [49], to induce apoptosis of the Jurkat leukemia T cell line [22] and to arrest the growth of human carcinoma cells [12].

LF levels are increased in nasal secretions of subjects with allergen-induced rhinitis [32]. The cellular source of both lactoferrin and lysozyme has been identified as serous cells of the submucosal glands [33]. Reports on the expression and presence of the protein in NP tissue and nasal secretions are rare and have not consistently demonstrated an increase [23, 43] or decrease [30, 31] in the LF level in polyp tissues. Additionally, no expression or downregulation of LF genes has been observed in nasopharyngeal carcinoma [42, 44]. In addition, despite higher LF expression in the submucosal tissue of NPs, the epithelial cells stained negatively for LF [43]. Hence, we assumed that the majority of reports demonstrated decreased LF levels in nasal secretions.

In view of these findings, we hypothesized that decreased expression of LF could be one of the etiological factors disturbing the local homeostasis of the nasal mucosa. Therefore, the aim of this study was to evaluate the effect of LF on the growth of NP fibroblasts and to characterize putative cell receptors for LF on cultured fibroblasts.

Materials and Methods

Reagents

All reagents were purchased from Sigma-Aldrich unless otherwise stated. TLR2 and TLR4 antibodies were from eBioscience, pyrogen-free heparin was obtained from SERVA, and bovine milk lactoferrin (BLF) was purchased from Morinaga Milk (Tokyo, Japan). Human milk lactoferrin (HLF) and iron-saturated human milk lactoferrin (HLF-iron) were from Sigma-Aldrich (cat. No. L0520 and L3770, respectively).

NP specimens

All subjects met the diagnostic criteria for chronic rhinosinusitis as established by the Task Force on Rhinosinusitis (AAO-HNS) [21]. Patients had been free of any medication for at least 4 weeks prior to surgery and had bilateral polyps in the nasal cavities identified by endoscopic examination and computed tomography (CT). The presence of comorbidities or smoking history was also cause for exclusion. NP were removed from the ethmoid during standard polypectomy or functional endoscopic sinus surgery in the ENT Department of the Wroclaw Medical University.

NP specimens were immediately disinfected with Betadine, rinsed in phosphate-buffered saline (PBS), cut into small pieces and placed into a sample tube containing 1 ml PBS. The tubes were directly transported on ice to the laboratory for further analysis. A part of each sample was fixed in 10% buffered neutral formalin, processed routinely, and embedded in paraffin wax for subsequent immunohistochemical examination to verify the diagnosis and to exclude other pathologies.

Informed consent was obtained from each patient. The study was approved by the Local Ethical Committee of Wroclaw Medical University.

Establishment of fibroblast cultures

NP biopsy samples were minced and incubated in a digestion mixture at 37°C for 2 h. The digestion mixture consisted of 5 ml of RPMI 1640 with 5% fetal calf serum (FCS), 0.2% clostridial collagenase type I and 0.005% deoxyribonuclease. When the dissociation of the tissue was complete, 0.5 ml of Hanks medium (Mg^{2+}- and Ca^{2+}-free) with 5% FCS and 25

mM EDTA was added for 15 min. After incubation, the cells were washed twice with Hanks' medium and subsequently cultured in flasks containing culture medium, which was composed of RPMI 1640 with 10% FCS, L-glutamine, sodium pyruvate, 2-mercaptoethanol and antibiotics. The medium was replaced every 6 days. For the experiments, the cells were detached from the culture flasks by treatment with 0.05% trypsin-EDTA followed by two washes with Hanks' medium.

Statistics

The data are presented as the mean ± SEM. All statistics were calculated using Statistica 7. The normality of the distributions and the homogeneity of the variance were tested. The differences between groups were analyzed using ANOVA, and Tukey's *post-hoc* test was applied. A level of $p < 0.05$ was considered to be significant.

Results

Growth inhibition of polyp-derived fibroblasts by various molecular forms of LF

Fibroblasts isolated from NPs obtained from patients with chronic rhinosinusitis were incubated for 6 days with various molecular forms of human lactoferrin (native and iron-saturated forms) and bovine (xenogeneic) LF in the concentration range of 1–25 μg/ml (Fig. 1). Dexamethasone (DEX) (50–0.05 μg/ml) served as a reference drug. Only data on the proliferation of NP fibroblasts isolated from three patients (two males and one female), randomly chosen from a group of six patients with chronic rhinosinusitis, are presented. The results showed that cell proliferation was inhibited, in a dose-dependent manner, by both human and bovine LF. To evaluate the effect of the iron saturation of LF on its ability to suppress fibro-

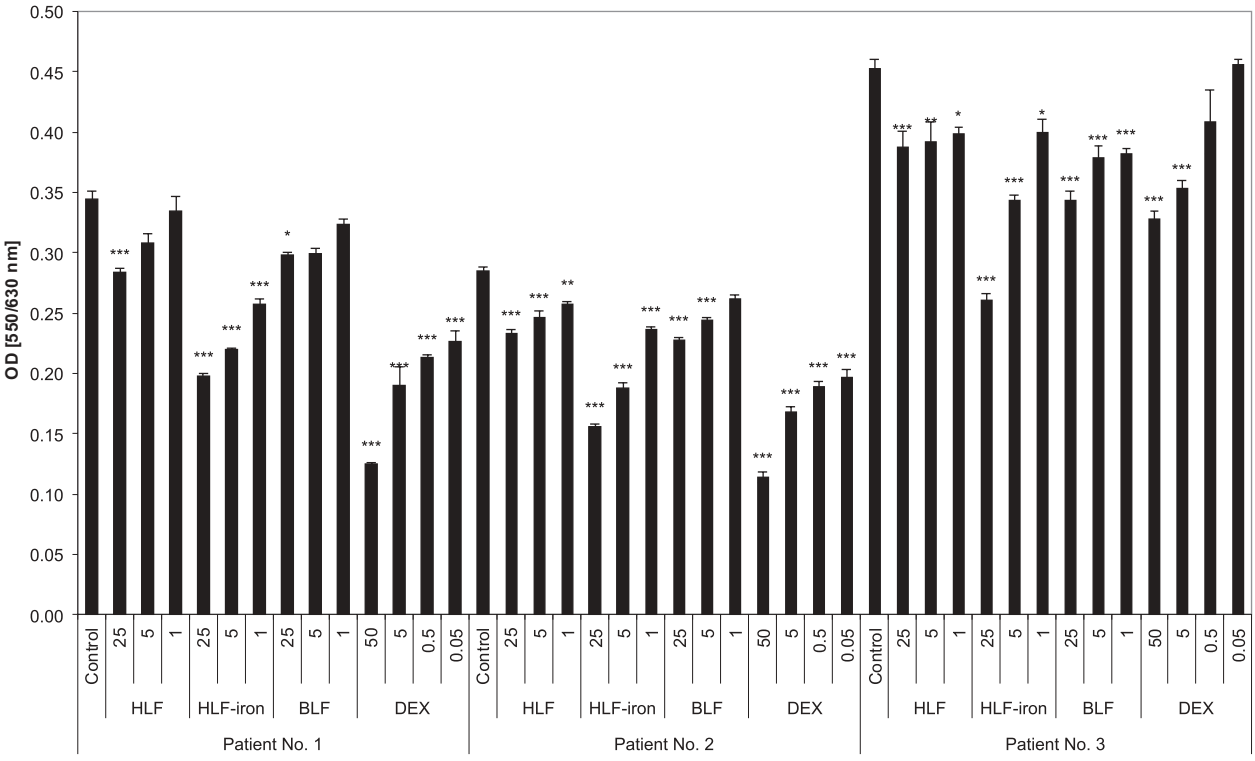

Fig. 1. The effect of lactoferrin (LF) on the growth of human fibroblasts *in vitro*. Fibroblasts isolated from nasal polyps of patients (n = 3) (5×10^4 cells/well) were incubated in the presence or absence of various concentrations of native or iron-saturated human milk lactoferrin (HLF) or bovine milk lactoferrin (BLF) for 6 days at 37°C. After the incubation period, the level of cell proliferation was determined using a colorimetric method (MTT) [16]. Values represent the means ± SEM of quadruplicate cultures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significantly different with respect to the control culture

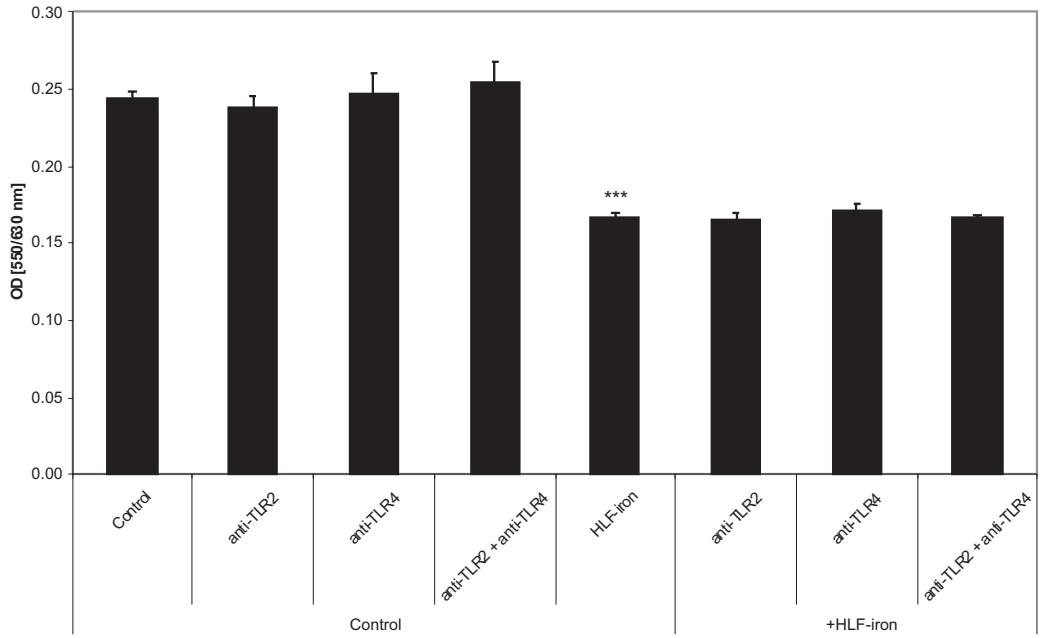

Fig. 2. TLR2 and TLR4 antibodies did not abrogate the inhibitory activity of iron-saturated human milk lactoferrin (HLF). Human fibroblasts (5×10^4 cells/well) isolated from the nasal polyps of patient no. 3, who suffered from chronic rhinosinusitis, were left untreated or were pretreated with 2.5 μg/ml of Ab to TLR2 or TLR4 (30') before the addition of iron-saturated HLF (25 μg/ml). After the incubation period, the level of cell proliferation was determined using a colorimetric method (MTT) [16]. Values represent the means ± SEM of quadruplicate cultures. *** $p < 0.001$ significantly different with respect to the control culture

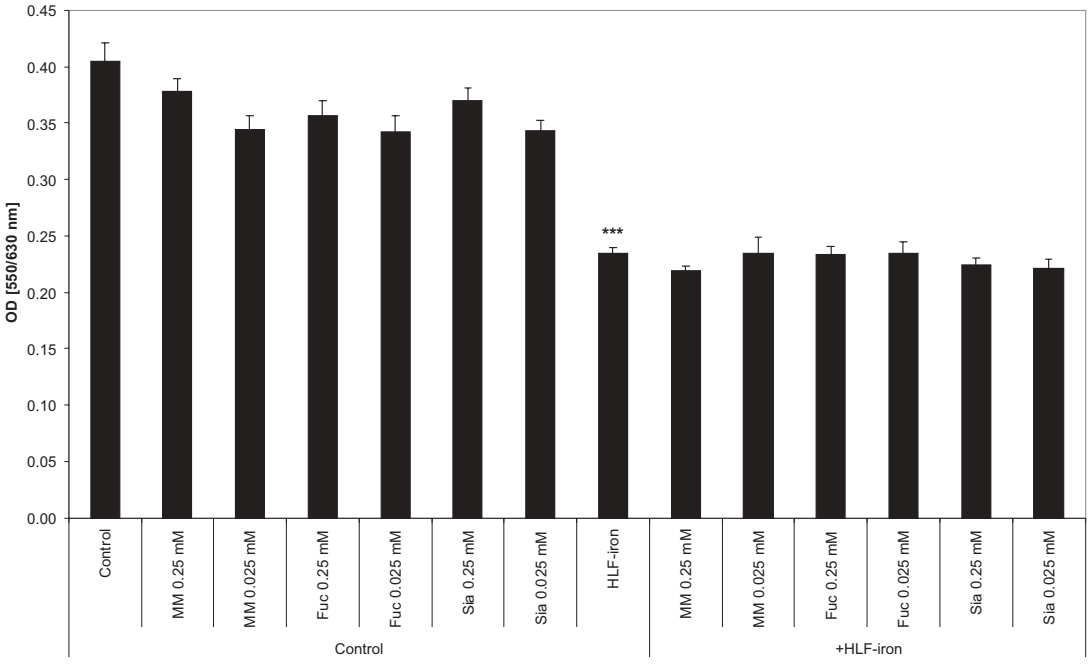

Fig. 3. Different monosaccharides had no effect on the inhibitory activity of iron-saturated human milk lactoferrin (HLF). Human fibroblasts (5×10^4 cells/well) isolated from the nasal polyps of patient no. 3, who suffered from chronic rhinosinusitis, were incubated in the presence or absence of 0.25 mM or 0.025 mM of different monosaccharides (α-methyl-D-mannopyranoside (MM), L-fucose (Fuc), sialic acid (Sia)) and iron-saturated HLF (25 μg/ml) for 6 days at 37°C. After the incubation period, the level of cell proliferation was determined using a colorimetric method (MTT) [16]. Values represent the means ± SEM of quadruplicate cultures. *** $p < 0.001$ significantly different with respect to the control culture

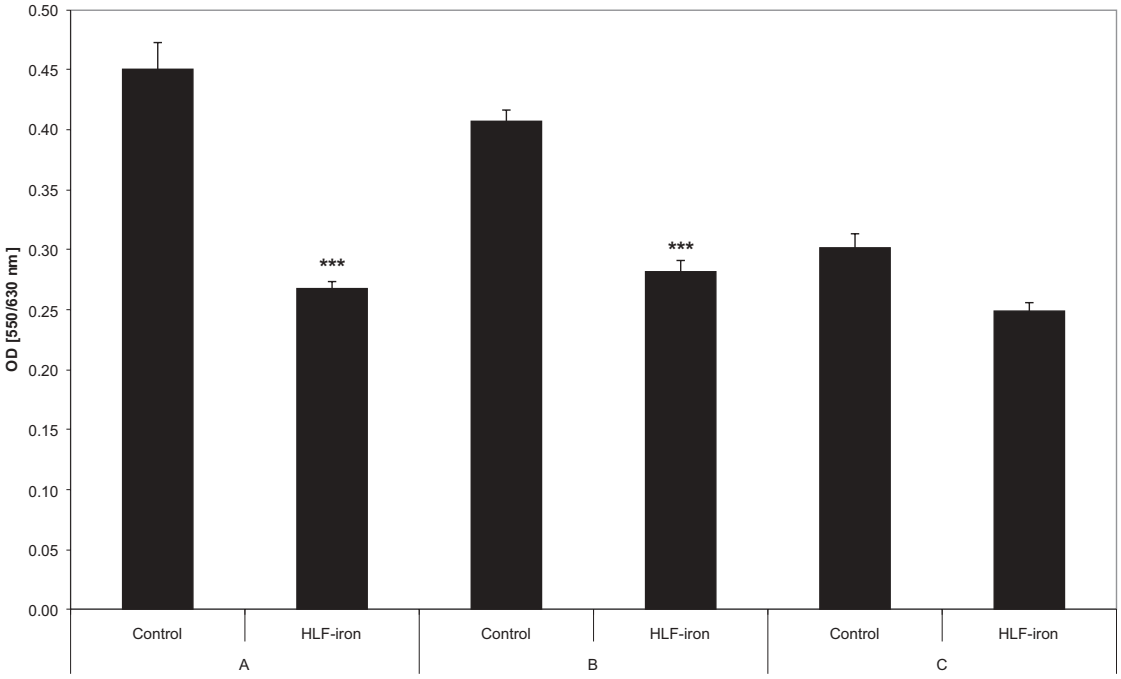

Fig. 4. Effect of sodium chlorate treatment of fibroblasts isolated from the nasal polyps of patient no. 3, who suffered from chronic rhinosinusitis, on the inhibitory activity of human milk-derived iron-saturated lactoferrin. Fibroblasts were (**A**) untreated, (**B**) preincubated with 20 mM sodium chlorate for 48 h, or (**C**) preincubated (48 h) and co-cultured with 20 mM sodium chlorate for next 6 days. Cells were incubated in the presence or absence of iron-saturated human milk lactoferrin (HLF) for 6 days at 37°C. After the incubation period, cell proliferation was determined using a colorimetric method (MTT) [16]. Values represent the means ± SEM of quadruplicate cultures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significantly different with respect to the control culture

blast proliferation, we compared the activities of native and iron-saturated lactoferrin from human milk in the fibroblast cultures. As shown in the figure, the inhibitory activity of human iron-saturated LF was distinctly higher than that of native LF. Figure 2 shows that addition to the cultures of anti-TLR2 or anti-TLR4 antibodies did not affect the inhibitory action of LF on fibroblast proliferation (only data on the proliferation of NP fibroblasts isolated from patient no. 3 for iron-saturated HLF are presented).

Characteristics of the LF receptor

In the next step, we attempted to characterize the nature of the fibroblast receptor for HLF. Figure 3 shows that addition of monosaccharides such as α-methyl-D-mannopyranoside (MM), L-fucose (Fuc), and sialic acid (Sia) to the cultures did not affect the inhibitory action of LF on fibroblast proliferation (only data on the proliferation of NP fibroblasts isolated from patient no. 3 for iron-saturated HLF are presented). In contrast, treatment of cells with sodium chlorate (Fig. 4) or with heparin (Fig. 5) abolished the inhibitory activities of LF.

Discussion

In this work, we demonstrated for the first time that LF, an important element of nasal exudates, can inhibit the growth of fibroblasts derived from NPs. The inhibition was stronger in the case of iron-saturated LF and involved a receptor containing heparan sulfate.

The concentration of LF in saliva and nasal secretions, which is increased in pathological conditions [23, 43], might indicate a requirement for this protein to counteract inflammatory reactions. It is conceivable that different mechanisms account for the antagonistic effects of LF that could directly or indirectly affect the formation of NPs. First, LF has been shown to reduce allergen-induced increases in cellular

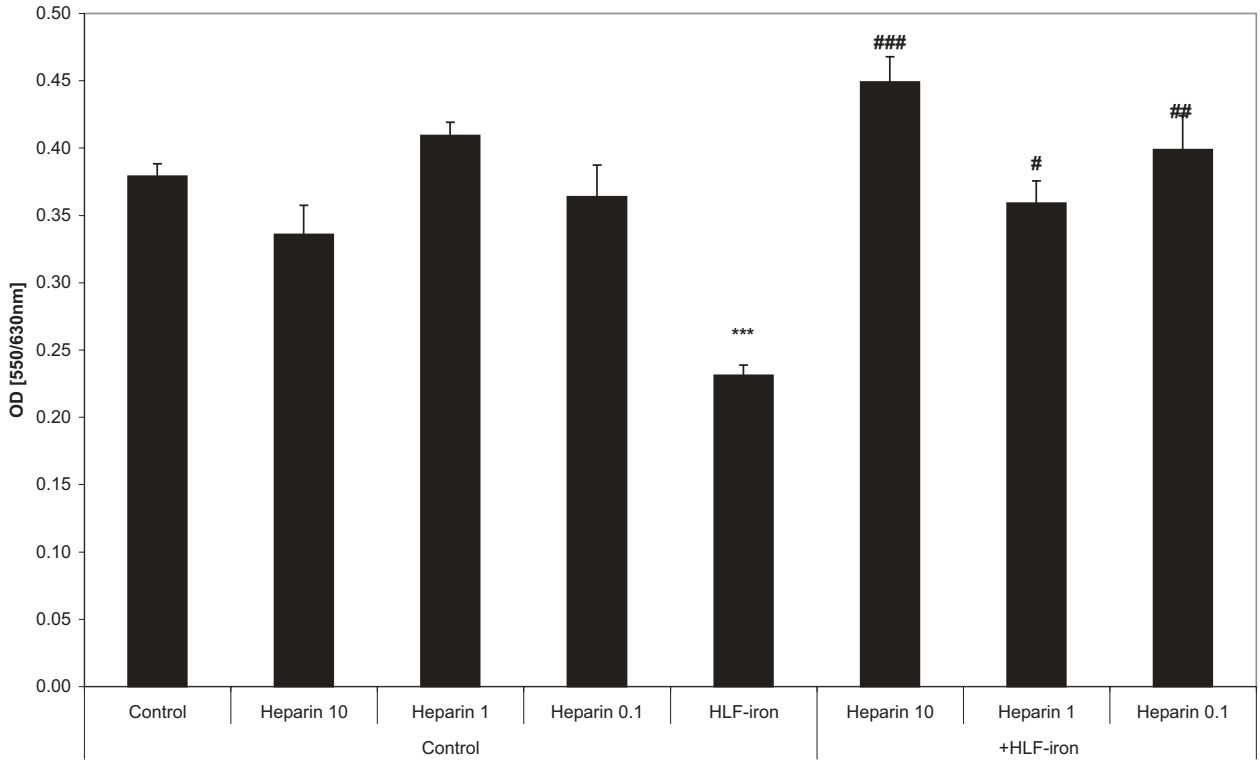

Fig. 5. Inhibition of the activity of iron-saturated human milk lactoferrin (HLF) by heparin. Human fibroblasts (5×10^4 cells/well) isolated from the nasal polyps of patient no. 3, who suffered from chronic rhinosinusitis, were incubated in the presence or absence of heparin (10–0.1 μg/ml) and iron-saturated HLF (25 μg/ml) for 6 days at 37°C. After the incubation period, cell proliferation was determined using a colorimetric method (MTT) [16]. Values represent the means ± SEM of quadruplicate cultures. *** $p < 0.001$ significantly different with respect to the control culture. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ significantly different with respect to the iron-saturated human lactoferrin (HLF-iron) culture (positive control)

reactive oxygen species and to reduce the accumulation of eosinophils in the airways and subepithelium of intranasally challenged sensitized mice [19]. Moreover, LF inhibits eotaxin-stimulated eosinophil migration, independent of the source of the LF (milk- or neutrophil-derived) [9]. Orally administered LF also decreased the eosinophil content in the circulating blood of cyclophosphamide-treated mice [2]. These findings are relevant to this study because the NPs were diagnosed as the eosinophilic type, so LF could have a potential therapeutic effect by inhibiting eosinophil infiltration. Second, the inhibition of pro-inflammatory cytokines [4] may play a role in the antiproliferative action of LF because the activity of basic fibroblast growth factor (bFGF) is stimulated by TNFα [39]. In addition, as discussed below, LF may compete with bFGF for the cellular receptor [14]. However, inhibition of metalloproteinase 2, which is involved in the pathogenesis of NP [7] by LF and acts as a metalloproteinase inhibitor *via* zinc chelation [26], is not likely because iron-saturated LF is more inhibitory than native LF. Although TLRs are present on fibroblasts [27] and LF may interact with TLRs [11], anti-TLR antibodies did not have an effect on LF activity in our study. This observation is important because LF binds endotoxins; thus, the potential presence of endotoxin in the nasal cavity could interfere with the inhibitory activity of LF.

In addition, inclusion of monosaccharides in the cell cultures did not inhibit LF activity, excluding a role of receptors specific for mannose or sialic acid in the inhibitory action of LF. In contrast, the pretreat-

ment of cells with sodium chlorate (an inhibitor of glucosamine sulfation) or the addition of heparin abolished the inhibitory effect of LF, suggesting that glycosaminoglycans containing heparan sulfate are the receptors for LF on fibroblasts. Interestingly, this type of receptor is also used by basic fibroblast growth factor [14], possibly suggesting a competitive mechanism for the inhibitory action of LF.

It is not quite clear why iron-saturated LF is a more potent inhibitor of cell proliferation than native LF. Binding of the glycosaminoglycan by human LF requires the cooperation of two distant sequences on the folded LF molecule, forming a cationic cradle as a binding site for chondroitin sulfate [25]. In contrast, iron binding is accompanied by a conformational change in the LF molecule [6]. It is, therefore, likely that higher saturation of LF with iron favors a more optimal conformation of the binding site.

This is a preliminary report that does not provide insight into the molecular mechanisms leading to the inhibition of fibroblast cell growth by LF. In the case of nasopharyngeal carcinoma, the inhibitory effects of LF are associated with cell cycle arrest at the G0/G1 phase, downregulation of cyclin D1 expression and modulation of mitogen-activated protein kinase activity [44]. The study on breast carcinoma cells revealed, on the other hand, growth arrest at the G1 to S cell cycle transition and modulation of the activity of key G1 regulatory proteins by LF [12]. Last, in leukemia T cells, LF caused apoptosis *via* regulation of c-Jun N-terminal kinase [22]. It is therefore likely that the mechanism of the inhibitory effect of LF on the proliferation of NP fibroblasts may share some of the molecular events mentioned above.

It is intriguing that LF does not provide sufficient protection against excessive cell proliferation despite the relatively high concentrations of this protein in nasal secretion [34]. It should be noted, however, that LF does not exist in body excretions as a free molecule, but, instead, it forms complexes with other proteins crucial for innate immunity [37].

The results of this study suggest that exogenously applied LF may have therapeutic applications in controlling NP growth, as in the case of glucocorticoids [41]. Such a presumption is further supported by the results of a clinical study demonstrating the inhibition of adenomatous colorectal polyps by orally administered LF [18]. To date, no side effects have been identified resulting from LF treatment and, more importantly, LF is a natural constituent of nasal secretions.

Acknowledgments:
The authors thank Ms. Zofia Sonnenberg for her excellent technical assistance. The study was supported by a statutory grant from the Polish Ministry of Education, No. 4/2008, for the Institute of Immunology, Polish Academy of Sciences, Wrocław, Poland and by the Ministry of Science and Higher Education (Poland), Grant No. 1043/B/P01/2007/33

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Received:
January 8, 2010.