Effects of Defensin and Lactoferrin on Functional Activity of Endothelial Cells *In Vitro*

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We studied the effects of antibacterial peptides and proteins (defensins and lactoferrins) on functional activity of endothelial cells *in vitro*: proliferative activity and adhesion of human endothelial ECV-304 cells to the matrix were evaluated. α-Defensin (NP-2) from rabbit neutrophils, total α-defensin (HNP 1-3) from human neutrophils, and lactoferrins from porcine neutrophils and human milk were studied. Defensins stimulated and lactoferrin in doses of 1-10 μg/ml inhibited proliferation and adhesion of endothelial cell. The stimulatory effect of defensins on proliferation and adhesion was reproduced in fibroblast culture. Lactoferrins did not modify proliferation of fibroblasts, but suppressed their adhesion. These data suggest that antibiotic proteins and peptides are prospective objects for the creation of drugs regulating angiogenesis.

Key Words: *defensin; lactoferrin; endothelial cells; angiogenesis*

Leukocytic cationic proteins and polypeptides are characterized by not only antibacterial, but also by immunoregulatory activity and serve as mediators of inflammation and cell-cell interactions [2]. Their participation in angiogenesis processes is disputed. For instance, proangiogenic [3] and antiangiogenic [5] effects of defensin administered to animals were reported. There are published data that lactoferrin (LF) inhibited vascular growth and suppressed endothelial proliferation *in vitro* [8]; however, opposite effects of LF were also observed [7].

We studied the effects of antibacterial peptides and proteins (defensins and LF) on functional activity of endothelial cells (EC) *in vitro*. Angiogenesis is usually studied *in vitro* by four tests reproducing the stages of vessel growth *in vivo*, specifically: EC migration, adhesion, and proliferation, and the formation of tubules resembling vascular lumen by these cells. Here we evaluated EC adhesion and proliferation.

MATERIALS AND METHODS

Two cell strains were used in the study: human endothelial cells ECV-304 and mouse fibroblasts L929 from collection of Cell Center of Institute of Cytology, Russian Academy of Sciences. Functional activity of ECV-304 cells was studied in RPMI-1640 without serum. The specificity of the observed effects was verified in L929 mouse fibroblasts.

Proliferative activity was evaluated by the MTT test. To this end, 10^4 cells were incubated with defensins or LF for 72 h at 37°C and 5% CO_2 and reduction of MTT dye (Sigma) was evaluated spectrophotometrically at λ=570 nm. Cell viability after incubation was ≥90% (according to trypan blue exclusion test).

Adhesion was evaluated as follows: 3×10^5 cells in 100 μl RPMI-1640 were placed into wells of a plate coated with 0.2% porcine skin gelatin (Sigma) and incubated for 1 h with the test agents, after

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which nonadherent cells were washed out with a buffer (pH 6.7-6.9; 500 mM imidasole, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA, 1 mM 2-β-mercaptoethanol, 4% polyethylene glycol 40,000) and adherent cells were stained with methylene blue, lyzed with 0.3 n HCl, and staining intensity was evaluated spectrophotometrically at λ=620 nm. Preliminary experiments showed that the intensity of cell staining depended on cell concentration in the well.

Rabbit α-defensin (NP-2) and human total α-defensins (HNP-1, HNP-2, HNP-3) were isolated from acetic acid extracts of rabbit and human neutrophils by ultrafiltration and HPLC. LF were isolated from porcine neutrophils and human milk by ion exchange chromatography and gel filtration. LF preparations were partially saturated with iron. The purity of the resultant preparations was controlled by electrophoresis and mass spectrometry.

The data were statistically processed using Student's *t* test.

RESULTS

The effects of the isolated antibacterial agents on proliferative activity of EC were compared with the effects of the following reference preparations: vascular endothelial growth factor (VEGF), a known mitogen for EC; TNF-α, a proinflammatory cytokine exhibiting angiogenic activity, but suppressing EC proliferation *in vitro*; phorbol ester, a synthetic analog of diacylglycerol (natural messenger), known for its activating effects. All three substances produced the expected effects: VEGF and phorbol ester stimulated and TNF-α suppressed proliferation of ECV-304 (Fig. 1). By contrast, TNF-α stimulated proliferative activity of fibroblast culture, similarly as phorbol ester.

In doses of 1-10 μg/ml defensins stimulated and LF suppressed proliferation of endothelial cells. The stimulatory effect of defensin was also reproduced in fibroblast culture, while the effect of LF on fibroblast proliferation was minor (Fig. 1).

The structure of human defensins HNP and rabbit defensins NP-2 is partially homologous, human defensins being less cationic and more hydrophobic. Our experiments revealed some differences in their action; for example, human defensin in a dose of 10 μg/ml suppressed proliferation of endothelial cells, while rabbit defensin exhibited no effect of this kind when incubated with cells.

Comparison of the effects of two LF on proliferation of human endothelial cell showed similar results, the preparation from human milk produced a more pronounced inhibitory effect in a wider dose range than porcine LF (Fig. 1), this indicating species specificity of this phenomenon.

Study of cell adhesion to matrix (gelatin-coated plastic) showed that TNF-α stimulated adhesion of ECV-304 cells and suppressed adhesion of L929 fibroblasts, while phorbol ester suppressed adhesion of ECV-304 cells and L929 fibroblasts (Fig. 2). Defensins stimulated adhesion of EC; human defensins did not stimulate adhesion of fibroblasts, while rabbit defensin in concentrations of 1-10 μg/ml stimulated adhesion of both cell strains. Addi-

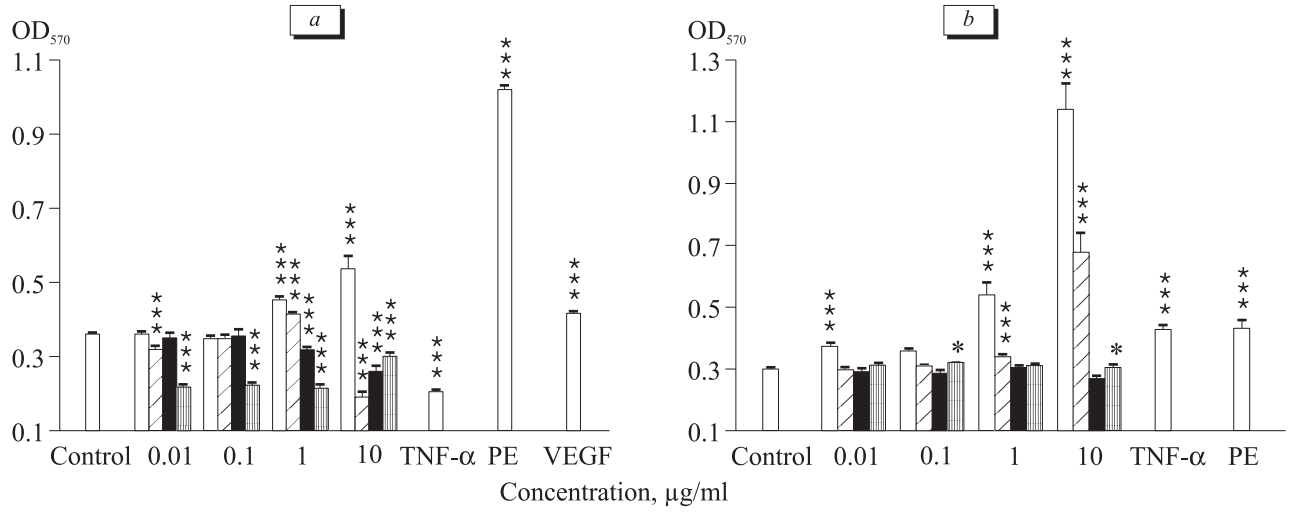

Fig. 1. Effects of cationic polypeptides on proliferation of ECV-304 (*a*) and L929 cells (*b*) over 72 h. Here and in Fig. 2: cells were incubated with NP-2 rabbit defensin (light bars), HNP human complex α-defensin (cross-hatched bars), porcine leukocytic LF (dark bars), human milk LF (vertically hatched bars) and in the presence of 100 U/ml TNF-α, 10 ng/ml phorbol ester (PE) or 10 ng/ml VEGF. Ordinates: optical density of solution. *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared to the control. Each experiment was carried out in 5 parallel samples and repeated 2-4 times.

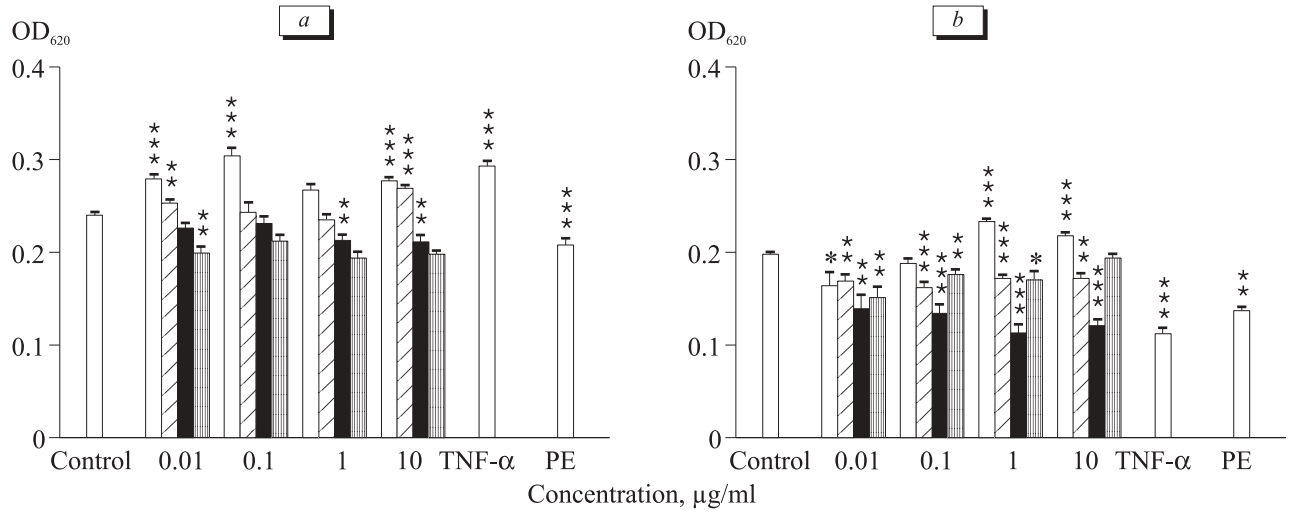

Fig. 2. Effects of cationic polypeptides on adhesion of ECV-304 (*a*) and L929 cell (*b*) to extracellular matrix after 1-h incubation.

tion of LF caused an opposite effect, both LF preparations suppressed adhesion of both fibroblasts and endothelial cells.

Opposite data on the role of defensins in angiogenesis can be found in literature. α-Defensins are low-molecular-weight cationic antibiotic polypeptides with a molecular weight of 3500-4000 Da present in neutrophilic azurophilic granules of humans and some animal species. Normal plasma concentration of defensins in humans in <40 ng/ml, but can increase to 170 μg/ml in patients with sepsis [9]. These concentrations are not cytotoxic *in vivo*, because defensins released into the plasma bind complement components and serine protease inhibitor proteins. During incubation in serum-free medium the cytotoxic effects of defensins start manifesting at a concentration of 10^{-5} M (about 35 μg/ml) [4]. The proangiogenic effect of defensin was revealed sufficiently long ago [3], but its mechanism is still not quite clear. On the one hand, defensins as cationic amphypathic compounds can promote cell-cell contacts through nonspecific electrostatic and/or hydrophobic interactions, on the other hand, they can specifically bind EC [6]. Human α-defensins inhibit EC adhesion and proliferation by modulating α5β1-integrin-mediated binding of these cells to fibronectin (the effects of defensins on adhesion to other matrix types, *e. g.* vitronectin, fibronectin, collagen, are negligible) [5]. In a previous study [6] higher concentrations of defensins were used. Hence, defensins in the concentrations used in our study can bind to EC *in vivo* and exhibit proangiogenic effects.

Lactoferrin (~77 kDa) is a cationic protein of specific granules of neutrophilic granulocytes; it is present in virtually all external secretions of humans and the majority of mammals. Normal LF content in donor plasma is 1 μg/ml, but it can increase more than 15-fold during infections [1]. Lactoferrin can bind metal cations of alternating valency, creating, for example, iron-deficient medium, which can underlie its bacteriostatic and antiproliferative effect [2].

Many cells *in vivo* interact with LF through specific receptors. Lactoferrin receptors were detected on activated lymphocytes, monocyte/macrophages, fibroblasts, intestinal epithelial cells, nonactivated platelets (LF binding to this receptor prevents platelet aggregation). It is unknown, whether analogous receptors are present on EC [11]. Detection of LF-specific binding sites is difficult, because LF due to its high cationic activity and heterogeneity of its polysaccharide residues can interact with cell membranes not only by the ligand-receptor mechanism, but also via nonspecific binding. However, in contrast to defensins, LF as a rule act as blockers of cell adhesion [10] and proliferation [8]. Our findings are in line with this assumption.

The results suggest that antibiotic proteins and peptides are prospective objects for creation of drugs regulating angiogenesis.

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