ORIGINAL ARTICLE

The incidence of β -defensin-1, 2, 3 in human healthy and chronically inflamed nasal and tonsillar mucosa

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Summary

The nasal and tonsillar mucosa are exposed to massive incursions of pathological microorganisms. One of the mechanisms known to prevent an invasion of pathogens is an endogenous synthesis of antimicrobial peptides, which include human β -defensins-1, 2, 3 (HBD-1, 2, 3).

The aim of this study was to demonstrate the occurrence of HBD-1, 2 and 3 in the human nasal mucosa and palatine tonsils in healthy tissues and during chronic inflammation (nasal polyposis with and without the colonization of *Staphylococcus aureus* and chronic tonsillitis) and to evaluate their incidence under varying conditions.

Another target was to compare the occurrence of human β -defensins in these two different entities; that is, in the nasal mucosa and in the palatine tonsil.

It was assumed that the incidence of HBD-1, 2, 3 was lower in tonsils than in nasal mucosa; however, inflamed samples of tonsils and nasal mucosa showed no difference in the production of HBD-1, 2, 3. The presence of all three subfamilies of HBD was significantly lower in nasal polyps with *S. aureus* positive than in the negative control.

Key words: β -defensins; nasal mucosa; palatine tonsil; chronic inflammation

INTRODUCTION

Both the nasal and tonsillar mucosa are exposed to massive incursions of pathological microorganisms present in inhaled air and swallowed with food.

Therefore it is important for the nasal and tonsillar mucosa to have effective mechanisms to fight against these pathogens. These defense mechanisms include epithelial integrity, lymphoid cells within the mucosa, mucociliary apparatus and antimicrobial substances in the lining fluid of nasal mucosa and epithelial integrity, lymphoid tissue and the secreted antimicrobial substances of the tonsils. If one or several of these mechanisms fail, pathogens can enter the body through the nasal or tonsillar mucosa.

Among the antimicrobial substances produced by the epithelial cells are the human β -defensins-1, 2 and 3 (HBD-1, 2, 3). β -defensins are endogenous antimicrobial peptides which are cationic peptides, and their function is to produce lysis of the plasmatic

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membrane and changes in the permeability of the plasmatic membrane. They are important components of innate immunity against bacteria, fungi and viruses. They are involved in the early immune response against pathogens. The HBD-1 is expressed constitutively and cannot be up-regulated by bacteria, fungi or other inflammatory stimuli (Dunsche et al. 2001, Po-Hsu and Shee-Yie 2004, Klüver et al. 2006); the expression of the others (HBD-2, 3) is induced by infectious challenges (Klüver et al. 2006). The production of HBD-2 and 3 is influenced by different types of pathogens. HBD-2 and 3 represent human defensins shown to be produced following stimulation of the epithelial cells with microorganisms (Schibli et al. 2002). HBD-2 exhibits a potent antimicrobial activity against gram-negative bacteria and *Candida*, and only bacteriostatic activity against *Staphylococcus aureus* (Claeys et al. 2003). Po-Hsu and Shee-Yiu (2004) note that HBD-2 has a bactericidal effect against a number of gram-negative and gram-positive bacteria including *S. aureus*. HBD-3 shows bactericidal activity against gram-negative as well as gram-positive bacteria, including *S. aureus* (Claeys et al. 2003); this is in contrast to the findings of Harder et al. (1997) who describe a strong bactericidal effect on gram-negative bacteria, a high antimycotic potency but only a weak bacteriostatic effect on gram-positive *S. aureus* in oral tissues. It was found that the highest concentrations of *S. aureus* are found immediately distal to the anterior hairy epidermis in a moist squamous epithelium devoid of hair, cilia and microvilli. This localization may be due to the lack of ciliary clearance of nasal fluid from this area, so its resistance to colonization depends largely on the intrinsic antimicrobial properties of the nasal fluid (Cole et al. 2001). A very interesting finding is the detection of different levels of HBD-2 and 3 in the inflamed nasal mucosa with and without *S. aureus*. HBD-2 and 3 were negligibly induced in nasal epithelial cells exposed to the nasal carrier strain of *S. aureus* in comparison to the non-carrier strain. It suggests that carrier strains of *S. aureus* retain a competitive advantage over non-carrier strains by delaying the host innate response to epithelial colonization and infection (Quinn and Cole 2007).

HBD-1 was expressed in all nasal tissue samples, at levels that did not differ significantly and HBD-2 was found in the nasal polyps but not in healthy nasal mucosa (Lee et al. 2002). Some other authors wrote that HBD-2 was detected only in several samples of the healthy nasal mucosa. It was predominantly localized in the surface epithelial cells with the strongest positivity in the basal layer (Po-Hsu and Sheen-Yie 2004). Claeys et al. (2003) found no up-regulation for HBD-2 and 3 in paranasal mucosa

in patients with chronic sinusitis or nasal polyposis compared with healthy nasal mucosa.

There are differing opinions as to the presence of β -defensins in tonsillar tissue. HBD-2, 3 have been demonstrated both in the surface epithelium and in the epithelium of the tonsillar crypts. The expression of HBD-2, 3 was confirmed in tonsillar tissue with no significant difference between idiopathic hypertrophic tonsillar disease and recurrent tonsillitis. The deep crypts and the excessive microorganical load and bacterial diversity in tonsils could explain the more pronounced expression of inducible antimicrobial peptides in this organ (Claeys et al. 2003). Some others found that significantly increased levels of HBD-2 were detected only in chronic inflamed tonsils (Weise et al. 2002). Ball et al. investigated very interesting results: the surface epithelium of tonsils from recurrent acute tonsillitis patients showed reduced amounts of antimicrobial peptides HBD-1 and 3, compared to healthy controls. It may increase these patients' susceptibility to infection (Ball et al. 2007).

The aim of this study was to demonstrate the occurrence of human β -defensin subfamilies 1, 2 and 3 in healthy human nasal mucosa and human palatine tonsils and during chronic inflammation (nasal polyposis and chronic tonsillitis). Findings in patients with nasal polyposis were divided into two groups – the first group of patients with a positive colonization of *Staphylococcus aureus* in the nasal mucosa and the second one with negative results of cultivation.

Another aim of this study was to compare the occurrence of human β -defensins in these two different localizations.

MATERIAL AND METHODS

Samples of clinically healthy nasal mucosa ($n = 10$) were obtained from the lower nasal turbinate of patients undergoing endonasal surgery for septal deviation. The samples of nasal polyps were obtained during clinically indicated endonasal surgery from patients with nasal polyposis ($n = 50$). The microbial cultivation from the nose was made before surgery. These patients were divided into two groups – the first group of patients with a positive finding of *Staphylococcus aureus* in the nasal mucosa ($n = 10$), the second one with the negative cultivation from nasal mucosa ($n = 40$). The samples of tonsils were collected during surgery from patients suffering from chronic tonsillitis ($n = 11$) or obstructive sleep apnea syndrome – these were clinically healthy tonsils ($n = 8$). The microbial cultivation from patients with

chronic tonsillitis was made before tonsillectomy. Various types of pathogens were found in the cultivations from the tonsils. For light microscopy the samples were fixed in 4% paraformaldehyde (PFA) in a phosphate buffer solution (PBS) (pH 7.4) for embedding to paraffin or snap frozen in liquid nitrogen and kept frozen at -80°C . Paraffin and cryostat sections were used for the detection of defensins (HBD-1, 2, 3) via the three-step immunoperoxidase methods. The detection on cryostat sections was carried out on 4% PFA in PBS-fixed frozen sections. The paraffin sections were deparaffined using xylene and 96% ethanol. The 2% fetal bovine serum (Biosera, UK) in PBS was used for 30 minutes to block non-specific binding of immunoglobulins. The next step of processing was incubation with a primary antibody [Anti-Rabbit-HBD-1 (Alpha-Diagnostic, Inc., USA) 1:400, Anti-Rabbit-HBD-2 (Peptide Institute, Inc., Japan) 1:200, Anti-Rabbit-HBD-3 (Orbigen, USA) 1:100]. This was carried out for 60 minutes at room temperature. After washing in PBS, the sections were incubated with biotinylated Goat-Anti-Rabbit IgG in PBS (Sigma-Aldrich, USA) 1:200 as the secondary antibody for another 30 minutes at room temperature. After rinsing in PBS for another 5 minutes – repeated three times – the sections were incubated with Vectastain ABC Elite kit peroxidase (VECTOR lab., USA) and diluted in PBS for ABC buffer (1:50) for 30 minutes at room temperature. The preparations, gently rinsed in PBS for five minutes were exposed to Diaminobenzidine (DAB) peroxidase substrate solution (DAKO Cytomation, Denmark) until the brown staining appeared. The nuclei were counterstained with hematoxylin for 5 seconds.

Antigen retrieval was performed after deparaffination in TRIS buffer base (pH 9.5) in a microwave oven (1 minute 560W and 5 minutes 240W). Possible activity of endogenous peroxidase was blocked by 70% methanol and 1% hydrogen peroxide for 10 minutes. The above mentioned procedures were performed on cryostat sections with exception of the antigen retrieval.

RESULTS

Clinically healthy nasal mucosa from inferior turbinate

The production of HBD-1 was higher in this localization than in polyps and tonsils. The reaction product was found in the glandular ducts, in the serous cells of the serous demilunes but not in the superficial epithelium. HBD-2, 3 were found in very

large amounts especially in the glandular serous cells and in the excretory parts. Precipitate was accumulated in the whole cytoplasm. HBD-3 was detected in high amounts in the whole cytoplasm of superficial epithelial cells as well as in the epithelial lining of the glandular ducts.

*Nasal polyps with occurrence of *Staphylococcus aureus**

The production of all three human β -defensins (HBD-1, 2, 3) was very low; they were detected only in several localizations of superficial pseudostratified columnar epithelium and in several glandular duct cells. The distribution of precipitate in the cytoplasm had a granular pattern.

*Nasal polyps without occurrence of *Staphylococcus aureus**

HBD-1 was detected irregularly in some serous glandular cells, cells of excretory ducts and in individual cells of the surface epithelium in very low amounts. HBD-2, 3 were found in large amounts especially in the serous and excretory parts of the seromucous glands and in the superficial epithelial cell cytoplasm. HBD-2 was present diffusely in the apical part of the cytoplasm of the epithelial cells and on the surface of this epithelium, HBD-3 had a granular pattern in the whole cytoplasm of the superficial epithelium and glandular ducts (Fig. 1).

Fig. 1. **The nasal polyp with negative cultivation.** The presence of HBD-3 in the superficial pseudostratified columnar epithelium, in the excretory duct of a seromucous gland and in the cytoplasm of serous cells. Immunoperoxidase reaction (DAB). Counterstained by hematoxylin.

Clinically healthy tonsils

A quite low but regular amount of HBD-1 was found especially in the stratified squamous epithelium covering. Very low levels of HBD-2 were proven in the healthy tonsillar epithelium especially in the stratum spinosum. HBD-3 was present in the superficial epithelium in higher amounts than HBD-2. It was found especially in the endothelial cell cytoplasm of small veins (Fig. 2).

Fig. 2. **The lymphoid tissue with small veins in clinically healthy tonsil.** The detection of HBD-3 in the endothelial cell cytoplasm of small veins. Immunoperoxidase reaction (DAB). Counterstained by hematoxylin.

Fig. 3. **A small branching vein in the lymphoid tissue of chronically inflamed tonsil.** HBD-3 is localized in the cytoplasm of endothelial cells. Immunoperoxidase reaction (DAB). Counterstained by hematoxylin.

Chronically inflamed tonsils

HBD-1 was detected in the stratum spinosum layer of the epithelium of crypts. HBD-2 was present in quite high levels in the superficial epithelium and occasionally in the marginal zone of lymphatic

follicles. The HBD-3 was proven in the highest level in the superficial epithelium of crypts in the marginal zone of lymphatic follicles and a very expressive positivity was found in the endothelial cell cytoplasm of small veins (Fig. 3).

DISCUSSION

It is thought that *HBD-1* gene is expressed constitutively and that the production of a peptide is not up-regulated by pathogens (Dunsche et al. 2001, Po-Hsu and Sheen-Yie 2004, Klüver et al. 2006). In the literature some authors have found that the levels of HBD-1 do not differ significantly in various tissue samples (Lee et al. 2002); another text asserts that HBD-1 is reduced in the tonsils of patients with recurrent acute tonsillitis (Ball et al. 2007). Our results show that HBD-1 was detected in larger amounts in healthy nasal mucosa than in the healthy tonsils. Chronically inflamed samples did not produce significant differences between nasal and tonsillar localization. Only in nasal mucosa with positive *S. aureus* was the presence of HBD-1 very low.

HBD-2 was found only in several samples of nasal mucosa (Po-Hsu and Sheen-Yie 2004), in polyps it was detected regularly (Lee et al. 2002). In tonsillar tissue the production of HBD-2 does not differ in healthy and inflamed samples (Claeys et al. 2003). In other publications, authors report significantly higher production of HBD-2 in inflamed tonsillar tissue in comparison to healthy controls (Weise et al. 2002). The expression of HBD-2 is induced by different types of pathogens (Schibli et al. 2002, Klüver et al. 2006). We focused our work on the production of HBD-2 during chronic inflammation in nasal and tonsillar mucosa.

From our results we can say that HBD-2 was found in all tissues examined but in variable amounts. It was proven in very high levels in healthy nasal mucosa as well as in nasal and tonsillar inflamed samples with the exception of polyps with positive *S. aureus*. From literature we know that HBD-2 is able to act against *S. aureus* (Weise et al. 2002, Claeys et al. 2003) but our results have proven only very low production of this defensin in case of colonization of nasal mucosa by *S. aureus*. In the healthy tonsils the production of this defensin was not proven.

According to the literature, *HBD-3* similar to HBD-2 was detected in healthy nasal mucosa as well as in nasal polyps. An up-regulation was not found in these two types of samples. HBD-3 was demonstrated

in both healthy and chronically inflamed tonsils (Claeys et al. 2003).

We investigated whether HBD-3 was present in healthy nasal as well as tonsillar tissues in larger amounts than HBD-2. In healthy nasal mucosa this defensin was found in very high levels. The production in chronically inflamed nasal and tonsillar tissues was quite high with the exception of polyps with positive *S. aureus*. Very interesting was the localization of HBD-3 in the endothelial cell cytoplasm of small veins especially in tonsils.

HBD-1 is involved in innate immunity; our results show that HBD-1 is present more in healthy nasal mucosa than in healthy tonsils. A possible reason is that in the innate immunity of nasal mucosa the lining fluid is more involved with the antimicrobial substances in comparison to tonsils where the most effective mechanism against pathogens are the cells of the immune system (lymphocytes, leucocytes etc.). Even when HBD-2 and 3 are included in the group of antimicrobial peptides up-regulated by pathogens, we found these defensins in very high amounts also in healthy nasal mucosa. These results show its possible contribution to innate immunity. Our results have proven up-regulation of HBD-2 and 3 after the invasion of pathogens into the nasal as well as tonsillar region. The localization of HBD-3 in the endothelial cell cytoplasm of small veins in tonsils is very interesting. It shows the possible transport of HBD-3 from the tonsillar region to the circulatory system or in the opposite direction. This result is very important because it shows that the possible effect of HBD-3 is not only in the place of origin but that it can be transported to any other region.

The colonization of the nasal mucosa with *S. aureus* is a very important matter. The effect of HBD-2 and 3 against *S. aureus* is known. Some authors refer the bacteriostatic (Claeys et al. 2003) or bactericidal (Po-Hsu and Sheen-Yie 2004) effect of these defensins to *S. aureus*. HBD-2, 3 are very important components of the defense against pathogens. But our results showed that in case of mucosal colonization with *S. aureus*, the cells expressed very reduced ability to produce HBD-1, 2, 3 so the organism's immune defense is weakened.

These results can be also explained by the principally different role the lymphatic compartment has in the tonsillar region which surely take place in the processes of antigen determination during alimentary passages in comparison to nasal mucosa which is exposed to very high amounts of airborne pathogens during every inspiration. The most effective manner in which the nasal mucosa can fight against this infection is by means of the antimicrobial peptides in the superficial lining fluid.

In summary, it can be concluded that HBD-1, 2, 3 are synthesized more intensively in healthy nasal mucosa in comparison in healthy palatine tonsil. There was no confirmation of any significant difference in the production of HBD-1, 2, 3 in the nasal polyps without the presence of *S. aureus* and in the chronically inflamed tonsils. Very low – nearly none – incidence of HBD-1, 2, 3 was detected in the nasal polyps with positive *S. aureus*. HBD-3 was found in the endothelial cell cytoplasm of the small veins in both healthy and chronically inflamed tonsils.

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