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ORIGINAL ARTICLE

Expression of human β-defensin -1, -2, and -3 in non-inflamed pseudocyst, mucoceles

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OBJECTIVES AND DESIGN: The expressions of human beta defensin-1 (HBD-1), -2 (HBD-2) and -3 (HBD-3) in non-inflamed pseudocysts such as mucoceles were investigated immunohistochemically in this study.
MATERIALS AND METHODS: Mucocele specimens were obtained from 21 patients. The expression of HBDs was studied immunohistochemically by using antibodies directed against HBD-1, -2, and -3. Statistical analyses were carried out on serial sections stained with antibodies.
RESULTS: Cells expressing HBDs were found in mucoceles. The expression of HBD-2 was observed in floating cells in all the specimens, whereas HBD-1 and HBD-3-expressing cells were detected in 93% and 73% of the mucoceles, respectively. The HBD-2 signal was the most intense and the HBD-3 signal intensity was weaker than that of HBD-1. HBDs were expressed in neutrophils and in other floating cells. Interestingly, the signal intensity and the population of positive cells located close to the centers of cysts were higher than those located in the peripheral areas of cysts.
CONCLUSION: The expression of HBDs was found even in non-inflamed pseudocysts such as mucoceles. These results suggest that an unknown mechanism not involved in biophylaxis for the expression of HBDs may exist.
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Keywords: HBD-1; HBD-2; HBD-3; mucocele; immunohistochemistry

Introduction

The human mucocele is one of the most common lesions that appear in the proper layer beneath the oral epithelium. Mucoceles are generally formed by rupture of an excretory duct of the minor salivary gland in the proper layer of the oral mucosa. A wall composed of connective tissue from the proper layer surrounds the mucous storage and many cells, such as neutrophils, lymphocytes, macrophages, etc. float in the mucoceles (Figure 1). Defensins are known as a biophylaxis factor prior to express immuno reaction (Barnathan *et al*, 1997; SchrÖder and Harder, 1999; Duits *et al*, 2002). Defensins are small cationic peptides divided into α- and β-defensins and many subtypes of them. Eight human α-defensins have been identified (thus far). Four types of α-defensins were found in neutrophilic azurophil granules, called human neutrophil defensin, HNP-1, HNP-2, HNP-3 and HNP-4 (Ganz *et al*, 1985; Ganz and Lehrer, 1994; Risso, 2000). The other two types of α-defensins were identified in Paneth's cells in intestinal crypts (Ouellette and Selsted, 1996). Human β-defensins are classified into four subtypes, HBD-1, HBD-2, HBD-3, and HBD-4. The first human β-defensin (HBD-1) was initially detected in plasma filtrates (Bensch *et al*, 1995) and was subsequently found in the duct cells of minor salivary glands (Sahasrabudhe *et al*, 2000), in human pancreatic, lung and urogenital tract epithelial cells (McCray and Bentley, 1997; Valore *et al*, 1998; Zhao *et al*, 1996). HBD-2 is expressed in mucosal epithelial cells (Harder *et al*, 1997).

The expression of these defensins generally occurs in the areas with infection or inflammation. For instance, HBD-2 is expressed in alveolar macrophages and monocytes stimulated by pro-inflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and γ-interferon (IFN-γ) (Duits *et al*, 2002). HBD-3 is expressed in keratinocytes and in lung epithelial cells. The expression of HBD-3 in these cells is enhanced upon stimulation with bacteria or TNF-α (Harder *et al*, 2001). We previously demonstrated the expression of HBD-2 from the upper spinous layer through the parakeratinized layer of the buccal mucosa in patients with oral candidiasis (Sawaki *et al*, 2002). In subjects with radicular cysts or odontogenic keratocysts with infections, the expression of HBD-2 has been detected in keratinocytes in the parakeratinized oral epithelium and in granular layers of cystic walls from

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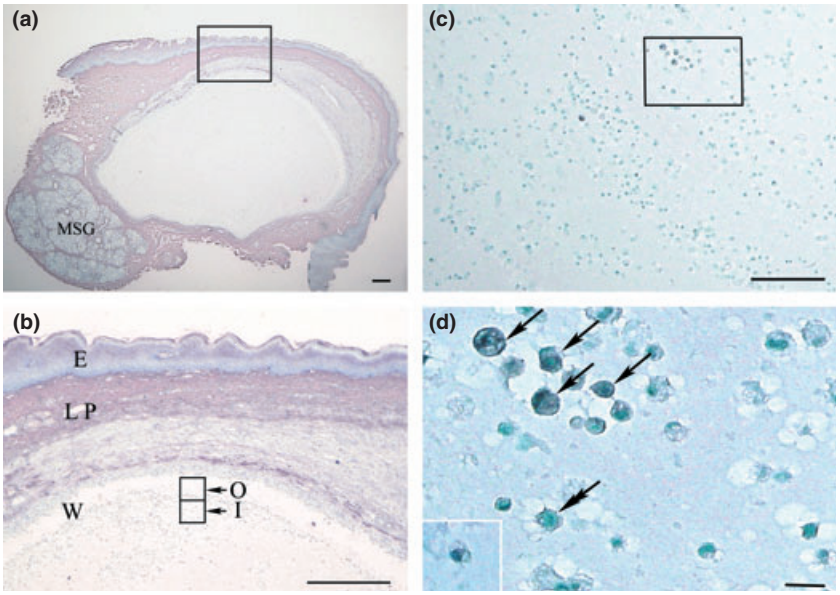

Figure 1 A microscopic view of a mucocele specimen under a low magnification (**a**). (**b**) Higher magnification of the square in (**a**). A temporary square in (**b**) was separated into two (50 μm × 50 μm) to designate inside and outside units. (**c, d**) Cells with typical positive signals in a mucocele immunostained with anti-HBD-2. (**d**) A microscopic view under a high magnification of (**c**). Arrows in (**d**) indicate positive cells. Double arrow indicates a typical vacuolated cell and inserted picture in (**d**) indicates a typical picnotic cell. MSG, minor salivary gland; E, epithelium; L P, lamina propria; W, cyst wall. Bars in (**a**) and (**b**) indicate 300 μm. Bar in (**c**) indicates 50 μm and bar in (**d**) indicates 15 μm. May-Grünwald was used as counterstaining in (**a**) and (**b**) and methyl green in (**c**) and (**d**)

odontogenic keratocysts. HBD-2 has also been detected in keratinocytes through upper spinous layers to granular layers from radicular cyst walls (Yoshimoto *et al*, 2004). However, the expression of HBDs has not been investigated in non-inflamed pseudocysts like mucoceles.

Experiments using human specimens are very important; however, these kinds of materials are hardly obtained. In this study, we were fortunate to have had a chance to report the expression and distribution of HBD-1, -2, and -3-expressing cells in mucoceles using immunohistochemical techniques and discuss a possible expression mechanism for HBDs in pseudocysts.

Materials and methods

All specimens were obtained from the lower lips of patients with mucosal cysts admitted to the Department of Oral and Maxillofacial Reconstructive Surgery, Okayama University Hospital, diagnosed with mucoceles (n = 21) (Figure 1a). The age of subjects ranged from 4 to 43 years, with 11 females and 10 males. Informed consent was obtained from each patient in accordance with the Guidelines of Okayama University Hospital.

Immunohistochemistry

After surgical operation, specimens were fixed with 10% neutral formalin for 6 h at room temperature and dehydrated by using graded concentrations of ethanol prior to embedding in Paraplast + (Sigma, St Louis, MO, USA). Serial sections (7 μm thickness) were cut and mounted on silane-coated slides. Immunohistochemical staining was performed according to the method of Mizukawa *et al* (2000) with minor modifications. Sections were irradiated on ice with 360 W microwave to activate antigens after deparaffinization. Irradiation was performed using three cycles of 2 min irradiation and 15 s interval to cool down by using a kitchen microwave oven. Polyclonal rabbit antibodies directed against HBD-1, HBD-2 and HBD-3 (1:900; Peptide Institute Inc., Osaka, Japan), IL-1β (Abcam, Tokyo, Japan) and TLR-2 (Biochain, Hayward, CA, USA) were used with ABC Reagent Kits (Vector Laboratories, Burlingame, CA, USA). Monoclonal mouse antibodies directed against TNF-α (Abcam) were also used. Antibodies against involucrin (Via Gramsci, Rome, Italy) and keratin (Cymbus Biotechnology, Hampshire, UK) that were generally detected in the epithelial cells were used as negative controls. To reduce nonspecific background signals, sections were blocked with normal serum. To block endogenous peroxidase activity, 1 M sodium azide was added to the color reaction medium. After immunoreactions were complete, sections were performed with methyl green. May-Grünwald's staining was also performed to distinguish the neutrophils. Cells were identified by their characteristic morphologic appearances.

Statistical analyses

To calculate the positive cell ratio of the immunoreaction, a temporary square frame (TS, 100 μm × 50 μm) was randomly planed on the microscopic field (Figure 1b) in each mucocele. After immunoreaction, it was possible to separate the signal intensities into two layers. So each TS was separated into two units (50 μm × 50 μm), the inside unit (arrow with I in Figure 1b, central part of the cyst) and the outside unit (arrow with O in Figure 1b, peripheral part of the cyst). Each TS is revealed in Figure 1b. The number of cells in 50 TSs was counted randomly from each mucocele sections for statistic analyses to compare signal intensities between both units. Student's t-tests were performed to analyze differences between inside and outside units and results are summarized in Table 1 and Figure 3.

Results

All specimens investigated in this study were diagnosed as true mucoceles at the University Hospital of Okayama University. Immunoreactions with anti-HBD-1, -2,

Table 1 Percentage of positive cells per mucocele[a]

	HBD-1	*HBD-2*	*HBD-3*
Outside	25.6 ± 4.6	28.0 ± 4.9	18.8 ± 3.1
Inside	32.8 ± 5.0	35.8 ± 5.1	25.2 ± 3.0
Total	29.1 ± 4.2	31.9 ± 5.1	22.2 ± 2.6
P	0.00045	0.00020	0.00009

HBD, human beta defensin.
[a]Comparison of the percentage of positive cells between inside and outside units. Distributions of positive cells for anti-HBD-1, -2, and -3 were statistically calculated in 50 inside and outside units per mucocele section. There is a significant difference between the percentage of positive cells of inside units and that of outside units with each antibody.

and -3 were performed on five serial slides from each specimen. Positive signals were not detected on the wall of the mucoceles but on floating cells in the mucoceles (Figure 2). HBD-2-positive floating cells (Figures 1d and 2) were found in all mucocele specimens, whereas 93% and 73% specimens also exhibited positive immunoreactivities for HBD-1 and HBD-3 in floating cells, respectively. The population of anti-HBD-2-positive cells per mucocele (31.9%) was the highest of all; however, the population of anti-HBD-3-positive cells (22.2%) was the lowest (Table 1). Anti-HBD-1, -2, and -3 signals were observed similarly in cytoplasms from neutrophils and lymphocytes or macrophages (Figure 2). The percentages of positive cells are summarized in Figure 3 and Table 1. The percentage of positive cells in the inside units of mucoceles (31.2%) was higher than that of outside units (24.1%). There was no significant difference in the cell density between the inside and outside units of mucoceles. The percentage of anti-HBD-2-positive neutrophils (15.6%) was the highest of all, whereas the percentage of anti-HBD-3-positive neutrophils (11.6%) was the lowest of all, both in inside and outside units (Figure 3b). Positive cells were observed in both inside and outside units. However, signals found in inside units were significantly higher than those found in outside units ($P < 0.05$, Figure 3b). No positive neutrophils for anti-involucrin or anti-keratin were found (Figure 3b). There was no significant difference for the positive ratios with anti-HBDs on neutrophils (Figure 3). We tried to detect expressions of IL-1β, TNF-α and TLR-2 to clarify the relationship between the expression of HBDs and them by immunohistochemistry. However, the positive signal for only TNF-α could be found in floating cells, in the epithelial cells of the excretory duct of the minor salivary glands and in the endothelial cells of capillaries around mucoceles (Figure 4a,b).

Discussion

Mucoceles are common lesions observed in oral epithelia without infection and inflammation, and are generally formed by rupture of an excretory duct of a minor salivary gland. After the mucocele is formed, the glandular structure of the minor salivary gland gradu-

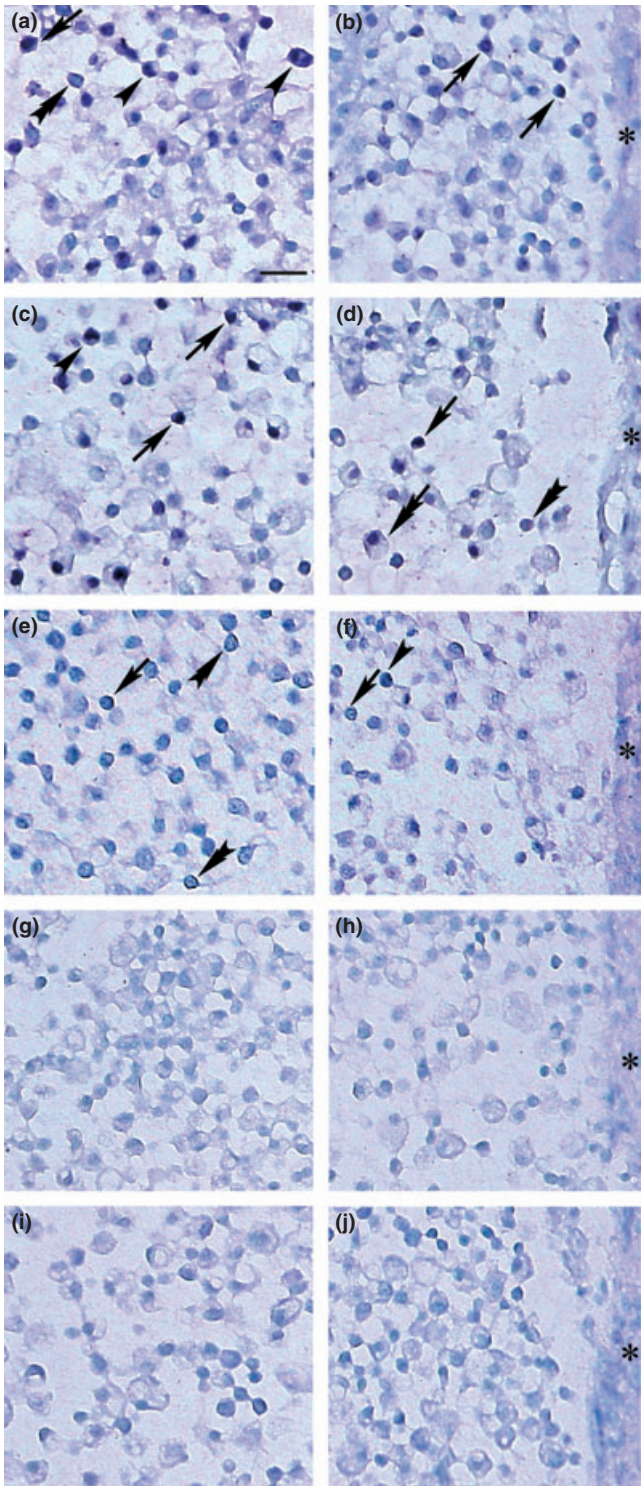

Figure 2 Localization of cells with positive signals of HBD-1 (**a, b**), HBD-2 (**c, d**), and HBD-3 (**e, f**) and control involucrine (**g, h**) and keratin (**i, j**). (**c**), (**e**), (**g**), and (**i**) indicate the micrographs of inside units. (**b**), (**d**), (**f**), (**h**), and (**j**) indicate the micrographs of outside units. Arrows in (**a**)–(**f**) indicate positive cells. Arrowheads in (**a**), (**c**), and (**f**) indicate positive neutrophils. Double arrowheads in (**a**), (**d**), and (**e**) indicate positive lymphocytes. Double arrows in (**d**) indicate macrophages. A May-Grünwald stain was used for counterstaining to distinguish the neutrophils. Magnifications from (**a**) to (**j**) are the same. Asterisks in (**b**), (**d**), (**f**), (**h**), and (**j**) indicate cyst wall. Bar in (**a**) indicates 25 μm

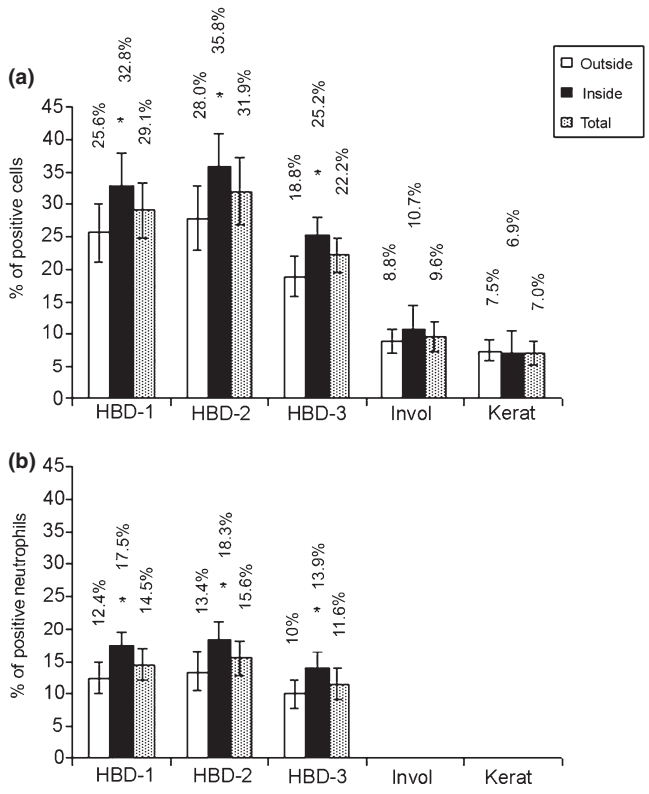

Figure 3 Histograms summarize the distribution of positive cells between central and peripheral parts of mucocele specimens investigated by using HBD-1, -2, and -3. (**a**) Percentage of all positive cells from all specimens of mucoceles. (**b**) Percentage of positive neutrophils. No neutrophil has positive signals for anti-involucrine and anti-keratin. $*P < 0.05$

ally disappears (Figure 1) and the wall composed of the connective tissue appears. The mucous storage increases, thereafter, in the mucocele. The infiltration of blood cells, neutrophils, or macrophages can be observed as they float in the lumen of pseudocysts, however, no inflammation was observed around the mucoceles (Figure 1). Immunohistochemical staining was performed on serial sections of mucoceles. We detected the expression of HBDs in floating cells in this study (Figures 1 and 2). Defensin is known as a biophylaxis factor and is usually detected in infected areas (SchrÖder and Harder, 1999), in the surface of the body (Abiko *et al*, 2001; Kimball *et al*, 2006; Poindexter *et al*, 2006) or the luminal epithelial cells and in human squamous cell carcinomas (Yoshimoto *et al*, 2003). However, the presence of these antimicrobial peptides in non-infected and non-inflamed pseudocyst like mucoceles has not yet been reported. The present study is the first report to demonstrate the expression of HBD-1, -2, and -3 in mucoceles. We analyzed the positive ratio of mucoceles and the signal intensity immunohistochemically in mucoceles to investigate their expressions and distributions. The expression of HBD-2 was observed in floating cells in all mucocele specimens, whereas the expressions of HBD-1 and HBD-3 were detected in 93% and 73% of specimens, respectively. The HBD-2 signal was the most intense and the signal intensity of HBD-3 was weaker than that of HBD-1. These results were almost similar to that of other reports (Yoshimoto *et al*, 2003; Wehkamp *et al*, 2003). Additionally, we calculated the percentage of positive cells for anti-HBD-1, -2, and -3 as shown in Figure 3a–c. The percentage of anti-HBD-2-positive cells was the highest, whereas anti-HBD-3 retained the lowest percentage (Table 1). The number of positive cells located in inside units was higher than that found in outside units. The production of HBD-2 is upregulated by cytokines such as TNF-α and IL-1β (SchrÖder and Harder, 1999), and also induced by TLR-2 (Hertz *et al*, 2003; Birchler *et al*, 2001) or by lipopolysaccharides (Nagy *et al*, 2006). It was reported that the concentration of TNF-α from subjects with salivary extravasation mucoceles is higher

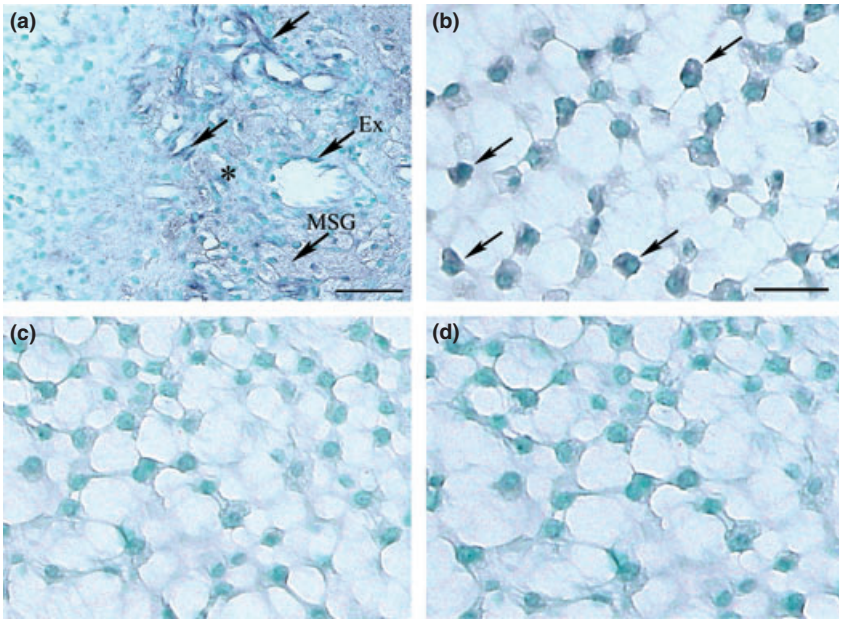

Figure 4 Immunolocalization of TNF-α (**a, b**) and the immunonegativity of IL-1 β (**c**) and TLR-2 (**d**) in the mucoceles. Methyl green was used as counterstaining. Arrows in (**a**) indicate the positive signals of TNF-α on the endothelial cells of capillaries and on the epithelial cell of the excretory duct of the minor salivary gland (arrow with Ex). Arrow with MSG indicates a degenerating minor salivary gland. Asterisk indicates cyst wall. Arrows in (**b**) indicate floating positive cells. No positive cell for IL-1 β or TLR-2 was found in the mucoceles. Bar in (**a**) indicates (50 μm), bars in (**b**), (**c**), and (**d**) indicate 25 μm

in the fluid of mucoceles than in saliva from Wharton's duct in the submandibular gland (Hoque *et al*, 1998). IL-1β and TNF-α were also detected in the cystic fluid of paranasal sinus mucoceles (Kariya *et al*, 2005). These reports indicate that the expression of HBDs might be induced by cytokines. In this study, we obtained only TNF-α signal in the excretory duct cells and in the endothelial cells of the capillary (Figure 4a); however, the percentages of positive cells for anti-HBD-1, -2, and -3 in neutrophils are low (Figure 3b). These results indicate that the expressions of HBDs in mucoceles might not be based on an inflammatory reaction.

The expression of HBD-1 has been reported in duct cells from normal minor salivary glands observed in mucoceles (Sahasrabudhe *et al*, 2000). The expression of HBD-1 has also been shown in neutrophils, leukocytes (Bensch *et al*, 1995), T-lymphocytes CD3, CD8, and macrophages (Wah *et al*, 2006). These reports indicate that the generation of HBD-1 might have occurred in floating cells in mucoceles. In this study, the expression of HBD-1, -2, and -3 was detected in the cytoplasm of neutrophils, lymphocytes and macrophages (Figure 2a,b). It was reported that the expression of HBD-1 is up regulated by IFN-γ in gingival keratinocytes (Joly *et al*, 2005) and in human skin (Sørensen *et al*, 2005). HBD-2 has been found in hyperplastic-stratified squamous epithelia in odontogenic keratocysts and radicular cysts (Yoshimoto *et al*, 2004). HBD-2 was also found in the cytoplasm of epithelial cells located through the upper spinous layer to the parakeratinized layer of the buccal epithelia from subjects with oral candidiasis (Sawaki *et al*, 2002), in T-lymphocytes cd3, cd8, and macrophages (Wah *et al*, 2006). The expression of HBD-2 was upregulated by TNF-α in patients with lichen planus (Abiko *et al*, 2002) and by IL-1β in keratinocytes (Liu *et al*, 2002).

HBD-2 is known as a specific chemoattractant for TNF-α-treated human neutrophils (Niyonsaba *et al*, 2004). It could be speculated that the strong expression of HBD-2 in mucoceles could be the consequence of an upregulation by cytokines such as TNF-α or IL-1β. HBD-3 expression was demonstrated in non-epithelial tissues, such as leukocytes, heart, and skeletal muscle (García *et al*, 2001). The expression of HBD-3 mRNA has also been reported in human squamous cell carcinomas (Yoshimoto *et al*, 2003). It was also reported that the expression of HBD-3 is upregulated by TNF-α, IL-1β, or IFN-γ in some keratinocyte cell lines (Harder *et al*, 1997, 2001; Seo *et al*, 2001). The expression of TNF-α has also been reported in human endothelial cells (Shanahan *et al*, 1989; Neuhaus *et al*, 2000). Thus, in the present study, HBDs in mucoceles could be due to the presence of the cytokines in/around mucoceles. Thus, we performed experiments to detect cytokines, IL-1β, TNF-α, and TLR-2 in mucoceles by immunohistochemical methods. However, only TNF-α could be detected and no signals were found for IL-1β and TLR-2 (Figure 4). Interestingly, we observed more intense immunoreactivities for HBD-1, -2, and -3 in inside units than those from outside units; however, the signals for TNF-α were mainly found around the mucoceles (Figure 4a,b). These results indicate that a system inducing system anti gradient of cytokines might exist in mucoceles.

In addition, anti-involucrin and anti-keratin antibodies were used as negative controls. However, cells indicated signals for these antibodies were found in mucoceles at a low percentage. Sometimes, dead cells indicate certain signals for antibodies as an artifact. Similarly, these kinds of signals has been observed in this study. In the case of HBD-2, co-cultures with the human squamous cell carcinoma cell line-4 (HSC-4) and normal human epidermal keratinocytes (NHEK) induced the cellular reproductive death of NHEK (Sawaki *et al*, 2003) by HBD-2. In the combination culture, both HSC-4 and NHEK expressed HBD-2; however, HSC-4 was resistant to HBD-2. This result suggests that HBD-2 has a biophylaxic role and may also play a role in the growth mechanism of cancer. Mucoceles sometimes include dead cells within their floating cell population (obviously pyknotic cells or vacuolated cells, Figure 1d). It could be speculated that the existence of dead cells is the consequence of HBD-2 expression in mucoceles. However, more data are required to better understand the mechanism of expression of HBDs in non-inflamed pseudocysts like mucoceles.

Author contributions

Toshio Sugahara and Tomoichiro Yamaai designed the study, Mac Keven Frederic and Yoshihiro Kaneda analysed the data, Mac Keven Frederic, Naoki Katase and Nobuyoshi Mizukawa drafted the paper and Hitoshi Nagatsuka and Mehmet Gunduz prepared the histologic study.

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