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ORIGINAL ARTICLE

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Differential expression of human beta-defensin 2 in keratinized and non-keratinized oral epithelial lesions; immunohistochemistry and in situ hybridization

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Abstract Human β-defensin(hBD)-2, an antimicrobial peptide, is produced by various epithelial cells. Because hBD-2 expression in the oral epithelium has not been assessed, we investigated its localization in normal oral epithelium and epithelial lesions. hBD-2 expression was studied using immunohistochemistry and in situ hybridization on formalin-fixed, paraffin-embedded tissue sections from 30 cases of squamous cell carcinoma and 6 cases of leukoplakia. Immunostaining for hBD-2 was more intense in hyperkeratinized than in ortho- or non-keratinized epithelium. In contrast, signals for hBD-2 mRNA were frequently stronger in non-keratinized epithelium than in hyper- or ortho-keratinized epithelium. The results suggest that keratinization in oral epithelium plays an important role in the biological function of hBD-2 both at the mRNA level and in the retention of the peptide in the epithelium.

Keywords Defensin · Squamous cell carcinoma · Oral · RT-PCR · Antimicrobial peptide

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Introduction

Oral epithelia are constantly exposed to a variety of microbial challenges. The epithelium functions as a mechanical and protective barrier to resist bacterial infection. The oral mucosa is a highly permeable tissue with regional variation [12]. Although infiltration of inflammatory cells into the epithelium also functions as an antimicrobial mechanism, this infiltrate is not universally observed under normal conditions. Antimicrobial activity of the oral epithelium itself may contribute to the prevention of bacterial infection. Recently, the β-defensin family of antimicrobial peptides has been detected in oral epithelium [1, 6, 9, 13].

The defensins belong to a group of antimicrobial peptides which are divided into two classes, the α- and β-defensins. Six α-defensins and two β-defensins have been identified in humans. Four α-defensins, designated HNP-1, -2, -3 and -4, are expressed in neutrophils, and the remaining two, designated HD5 and HD6, are found in intestinal Paneth cells [3, 5]. The α-defensins have a broad spectrum of activity, killing Gram-negative and Gram-positive bacteria, fungi, and enveloped viruses. Human ß-defensin (hBD)-1 is expressed by the epithelial cells of many organs in humans, including the pancreas, kidney, salivary gland, trachea, and oral epithelium, and it is also effective for killing both Gram-negative and-positive bacteria [2]. The second type of hBD, hBD-2, has recently been identified [4]. hBD-2 is highly effective in killing Gram-negative bacteria, and its expression has been detected in the epithelial cells of the skin, lungs, uterus, trachea and, oral mucosa [1, 9, 11, 13]. hBD-1 is constantly produced, whereas the expression of hBD-2 is transcriptionally inducible by various proinflammatory agents such as cytokines and bacteria [1, 9, 13]. Therefore, hBD-1 may influence normal epithelial interactions with the commensal flora, and hBD-2 may participate in the host defense response to enteric microbes [10].

We have previously shown that oral epithelial cells, including primary cultured cells from gingiva and several kinds of squamous cell carcinoma (SCC) cell lines, expressed hBD-2 mRNA. [1]. Normal epithelial cells expressed hBD-2 mRNA, and this was up-regulated by stimulation with lipopolysaccharides (LPS) and tumor necrosis factor (TNF)-α. Levels of expression of hBD-2 in carcinoma cell lines varied greatly and, in some cell lines, were up-regulated by stimulation [1]. Although hBD-2 is very likely to play a role in protecting oral epithelium from bacterial infection, the expression of hBD-2 in oral epithelium in vivo has not been demonstrated thus far. In the present study, we investigated the localization of hBD-2 at both protein and mRNA levels on tissue sections of oral epithelium and oral lesion, including SCC and leukoplakia.

Materials and methods

Samples

Thirty cases of SCC with various differentiated features, six cases of leukoplakia, and normal epithelium adjacent to the lesions were used in the present study.

Immunohistochemistry

The surgical specimens and their surrounding normal tissues were fixed with 10% buffered formalin and embedded in paraffin. Deparaffinized sections were soaked in methanol containing 2% H_2O_2 to block endogenous peroxides. Sections were washed in phosphate buffered saline (PBS) and incubated at 4°C overnight with a primary antibody. Polyclonal rabbit anti-hBD-2 antibody (Peptide Institute, Inc. Osaka, Japan) was used as the primary antibody at a 1:4000 dilution. After sections were rinsed in PBS, a streptavidin-biotin peroxidase complex kit [SAB-PO (G) kit; Histofine, Nichirei, Japan] was used on the sections according to the manufacturer's instructions. Sections were immersed in 3,3′-diaminobenzidine (DAB) and H_2O_2 for 5 min at room temperature (RT), rinsed in distilled water, dehydrated, and mounted in Malinol. As a negative control, normal rabbit serum was used instead of the primary antibody in the same procedure.

Subcloning of complimentary DNA for hBD-2

We have previously confirmed that the SCC-9 cell line, derived from oral SCC, expresses hBD-2 mRNA [1]. Total RNA was extracted from SCC-9 cells using the acid guanidine thiocyanate/phenol–chloroform method, using a total RNA isolation reagent (Trizol Reagent, GIBCO BRL). For reverse transcriptase polymerase chain reaction (RT-PCR), 1 μg of total RNA was reverse-transcribed (Superscript reverse transcriptase GIBCO BRL) according to the manufacturer's instructions, using Oligo$(dT)_{12–18}$ primers (GIBCO BRL). The RT products were amplified using a PCR kit (AmpliTaq Gold with GeneAmpR 10×PCR buffer, Perkin Elmer, Applied Biosystems, Forster City, Calif.) and a thermocycler (Takara PCR Thermal Cycler MP, Osaka, Japan) according to the manufacturer's protocol. The primers were designed based on the complimentary (c) DNA sequences for hBD-2 [4]. The sequence of the forward primer was 5′-CCCAGCTTCCAGCCATCAGCCATGAGGGT-3′, which contained an additional recognition site of *Hind*III. The sequence of the reverse primer, containing an *Eco*RI recognition site, was 5′-CGGAATTAGGAGCCCTTTCTGAATCCGCA-3′. The PCR process consisted of 45 cycles, each cycle including 1 min of denaturing at 94°C, 1 min of annealing at 58°C and 1 min of primer extension at 72°C. The final extension step was for 9 min at 72°C.

After purification of the PCR product of approximately 280 bp using a PCR clean kit (Qiagen, Hilden, Germany), the product was cut out with two restriction enzymes, *Eco*RI and *Hind*III (GIBCO BRL). The digested product was electrophoresed on 1% agarose gels at 90 V (Bio-Rad, Hercules, Calif.). The bands containing the cDNA were cut, and the cDNA was extracted from the gel using a DNA recovery kit (Worthington Biochemical, Lakewood, N.J.).

The cDNA for hBD-2 was inserted into pT7T3-a18 (GIBCO BRL), transformed into DH-5α competent cells (GIBCO BRL), amplified, and isolated by using a DNA Extraction Maxi Kit (Qiagen). The plasmid containing cDNA for hBD-2 was linearized with restriction enzymes: *Hind*III and *Eco*RI for the sense and anti-sense RNA probe, respectively. Sense and anti-sense RNA probes were transcribed in vitro from the linearized plasmid by use of digoxigenin-labeled uridine triphosphate (UTP) and T3 or T7 polymerase according to the manufacturer's manual (Boehringer Mannheim, Mannheim, Germany).

In situ hybridization

The deparaffinized sections were pretreated with 10 μg/ml proteinase K and then treated with a triethanolamine buffer (pH 8.0) containing 0.25% acetic anhydride. The sections were hybridized with digoxigenin (DIG)-labeled antisense RNA probes for hBD-2 for 16 h at 45°C. Stringent washes were performed for 60 min at 45°C with 50% formamide in 2× sodium saline citrate (SSC), and the slides were treated with 20 μg/ml of RNase-A (Boehringer Mannheim, Mannheim, Germany) for 30 min at 37°C. The sections were incubated with anti-DIG antibody coupled to alkaline phosphatase (1/1000 dilution, Boehringer Mannheim) for 1 h at RT. Alkaline phosphatase was detected by incubation with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium (NBT) for 4 h at RT, as indicated in the Boehringer Mannheim DIG Detection Kit manual, except for the addition of levamisole (Sigma, St.Louis, Mo.). As a negative control, we used sense RNAs for hBD-2 instead of antisense RNAs, or omitted the antisense RNA probes under otherwise similar conditions for in situ hybridization.

Results

In oral epithelium, the hard palate, gingiva, and dorsal tongue are keratinized, but the oral floor and buccal region are non-keratinized. In normal oral epithelium, immunohistochemical staining for hBD-2 was weakly positive in the keratinized layers of the stratified squamous epithelium (Fig. 1a) and was negative in the non-keratinized epithelium. In cases of leukoplakia, hBD-2 stained intensely in both the hyperkeratinized layers and the granular layers (Fig. 2a). In SCC, expression of hBD-2 was heterogeneous in location and intensity, being mainly observed in the keratinized layer of epithelial pearl, where the staining was as intense as in leukoplakia (Fig. 3a and Fig. 4a). The areas around the keratinization corresponding to the granular layer often gave a strong signal. However, no hBD-2 expression was observed in poorly differentiated SCC (Fig. 5a). In addition, two cases of well-differentiated SCC failed to express hBD-2 even in the area around the keratinization.

By means of in situ hybridization, the expression of hBD-2 mRNA was detected in upper spinous layers of non-keratinized squamous epithelium in normal oral epithelium (Fig. 6a and Fig. 2b). Unlike immunostaining,

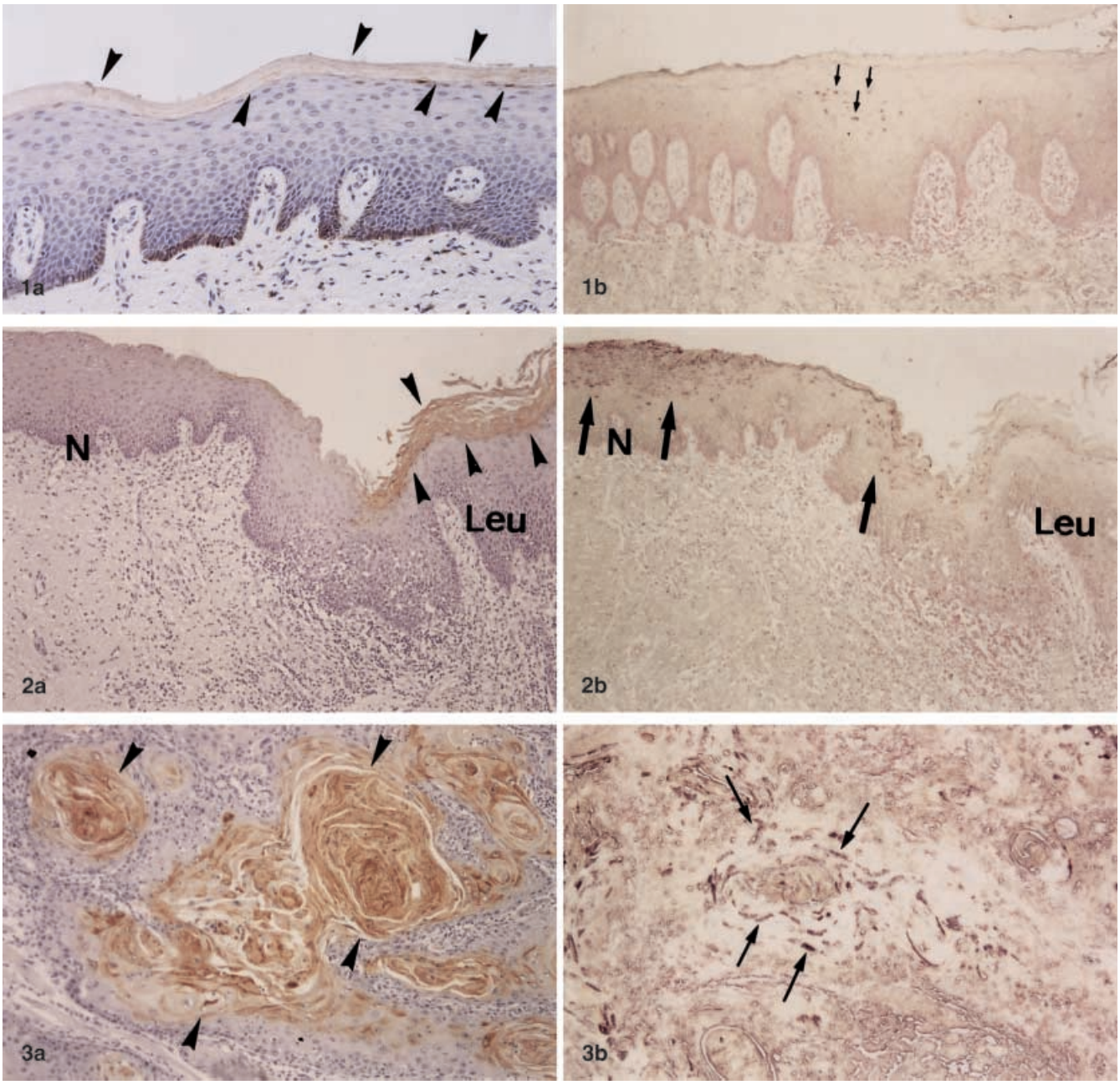

Fig. 1 Immunohistochemical staining of human β-defensin 2 (hBD-2; **a**) and in situ hybridization of hBD-2 (**b**) in the palatal mucosa with keratinization. The keratinized layer is weakly positive for hBD-2 peptide (**a**; 120), and hBD-2 mRNA signals are faint (**b**; ×70)

Fig. 2 Immunohistochemical staining of human β-defensin 2 (hBD-2; **a**) and in situ hybridization of hBD-2 in the area at the border between non-keratinized normal epithelium and leukoplakia in buccal mucosa (**b**). The non-keratinized area in the normal epithelium shows a weakly positive reaction for hBD-2 peptide but an intense signal for hBD-2 mRNA. In contrast, the area of leukoplakia shows a strongly positive reaction for hBD-2 peptide in the hyperkeratinized layer but a weak signal for hBD-2 mRNA (**a, b**; ×60). *N* normal non-keratinized epithelium; *Leu* leukoplakia with hyperkeratinized epithelium

Fig. 3 Immunohistochemical staining of human β-defensin 2 (hBD-2; **a**) and in situ hybridization of hBD-2 (**b**) in squamous cell carcinoma (SCC) with well-differentiated type. The immmunostaining for hBD-2 is mainly observed in the keratinized layer of epithelial pearl, and the signal for hBD-2 mRNA is mainly observed at the areas around the epithelial pearl. The areas around keratinization corresponding to the granular layer often show strong signals for hBD-2 at both the protein and mRNA level (**a, b**; ×70)

the hBD-2 signals in keratinized squamous epithelium were weaker than in the non-keratinized layer (Fig. 1b). The leukoplakia with hyperkeratinization yielded positive signals in limited numbers of cells in the granular layers (Fig. 2b). The extent of hBD-2 expression in SCCs varied widely, and the signals were mainly observed at the areas around the epithelial pearls and at the granular layer (Fig. 3b and Fig. 4b). No hBD-2 signals

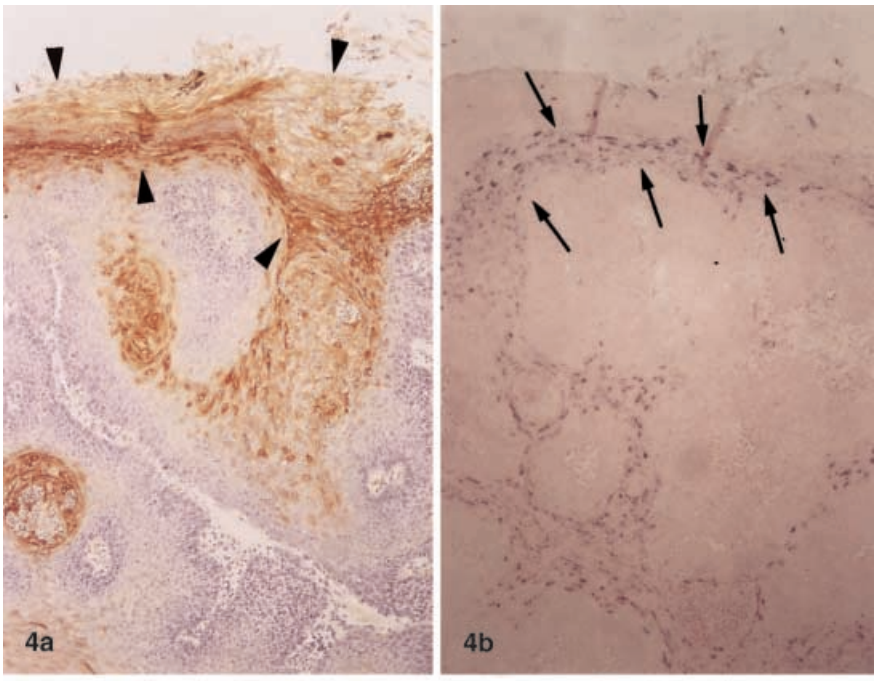

Fig. 4 Immunohistochemical staining of human β-defensin 2 (hBD-2; **a**) and in situ hybridization of hBD-2 (**b**) in verruous squamous cell carcinoma (SCC) with hyperkeratinization. The hyperkeratinized and granular layers shows a strongly positive reaction for hBD-2 peptide (**a**; ×60), and the signals for hBD-2 mRNA is observed at the granular layer (**b**; ×60)

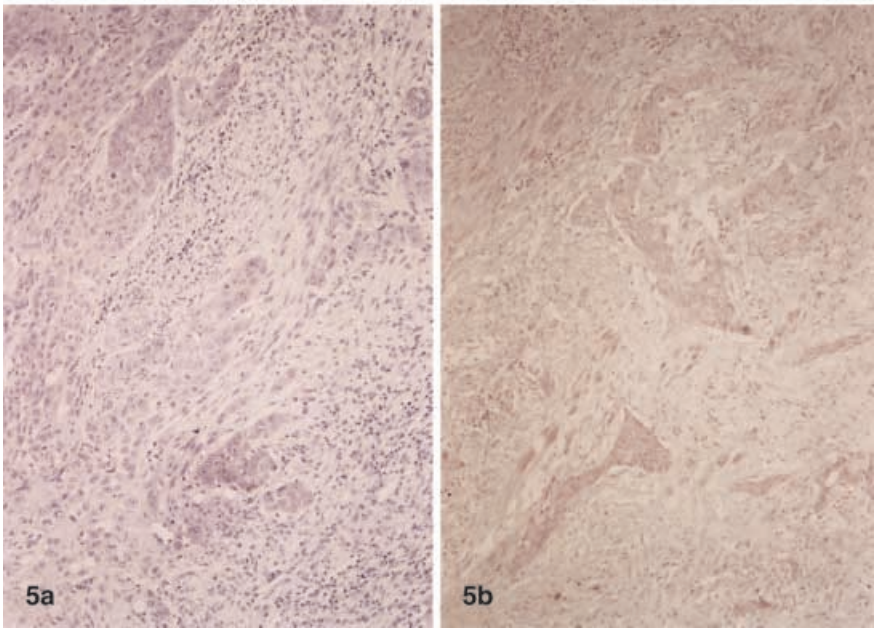

Fig. 5 Immunohistochemical staining of human β-defensin 2 (hBD-2; **a**) and in situ hybridization of hBD-2 (**b**) in squamous cell carcinoma (SCC) with poorly differentiated type. Neither immunohistochemistry nor in situ hybridization shows hBD-2 expression in a poorly differentiated type of SCC (**a, b**; ×60)

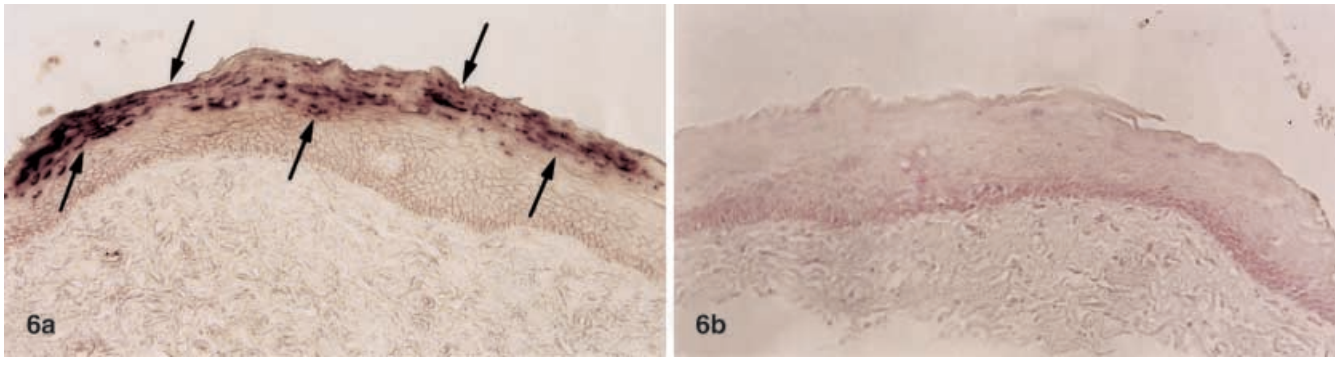

Fig. 6 In situ hybridization of human β-defensin 2 (hBD-2) in the buccal mucosa with non-keratinized epithelium. hBD-2 mRNA is detected in upper spinous layers in non-keratinized normal epithelium by means of in situ hybridization (**a**; ×130). For the hBD-2 sense probe, no positive signal is observed (**b**; ×130)

Table 1 Expression pattern of human β-defensin 2 protein and mRNA. *hBD-2* human β-defensin 2, *SCC* squamous cell carcinoma, – none observed or negative, + positive, ++ strongly positive, ~ variation in staining

	hBD-2 protein	hBD-2 mRNA
Normal epithelium		
Non-keratinized (oral floor, buccal mucosa)	–	– ~ ++
Keratinized (oral palate, gingiva)	– ~ +	– ~ +
Leukoplakia (hyperkeratosis)	+ ~ ++	– ~ +
SCC		
(keratinized area)	+ ~ ++	+ ~ ++[a]
(non-keratinized)	–	–

[a] Two cases of SCC with keratinization did not express hBD-2 at either protein or mRNA levels

were observed in the poorly differentiated type of SCC and in two cases of SCC without immunostaining for hBD-2 (Table 1).

Discussion

In this study, we provide the first report on the distribution of hBD-2 peptide and mRNA in normal oral epithelium and oral epithelial lesions. Our findings document stronger immunoreactions of hBD-2 in hyperkeratinized epithelia, despite non-keratinized epithelium yielding more intense signals for hBD-2 mRNA. In keratinized stratified squamous epithelium, the boundary of the granular and keratinized layer acts as a barrier to penetration of tracers, such as ovalbumin [12]. Therefore, the deposition of hBD-2 peptide at the boundary in keratinized epithelium resulted in an intense immunoreaction in the keratinized layer. hBD-2 is a low molecular weight peptide [11]. Therefore, in non-keratinized epithelium without such a boundary, the hBD-2 may easily diffuse out even if the cells actively produce and secrete the peptide. We hypothesize that the non-keratinized epithelia constantly express hBD-2 mRNA in order to maintain high levels of the peptide in their stratified layers for prevention of bacterial infection.

A previous paper showed that lesional psoriatic scale, a non-infectious inflammatory skin disease with keratinization, contained a large amount of hBD-2 [7]. Our present results showing strong expression of hBD-2 in hyperkeratosis are perfectly consistent with this finding [7]. In contrast, normal skin epidermis exhibiting orthokeratosis did not express either hBD-2 peptide or mRNA unless inflammation was present [7]. In our study, however, orthokeratinized oral epithelium showed some hBD-2 immunoreactivity and mRNA signals although the level of expression was lower than in epithelium with hyperkeratinization. The discrepancy may be due to different antibodies and probes used in each study or to differences in the environment of the skin relative to the oral cavity, the latter being constantly in contact with saliva and commensal flora.

SCC with keratin pearl formation tends to exhibit a more intense expression of hBD-2 at both peptide and mRNA levels. Most SCC is accompanied by severe inflammation, i.e., interstitial inflammatory reactions around the nests. Inflammation induces the expression of hBD-2 in certain kinds of epithelial cells [7, 8]. Thus, the expression of hBD-2 in SCC may be induced by some factors derived from the inflammatory cells. In contrast, we have previously reported that the level of hBD-2 mRNA in some oral carcinoma cell lines was extremely low and was not inducible by inflammatory cytokines, such as TNF and interleukin (IL)-1β, suggesting that hBD genes in those carcinoma cell lines could be mutated [1, 4, 5, 9, 11]. In the present study, neither hBD-2 peptide nor mRNA was detected in a poorly differentiated type of SCC. These cases may be due to the mutation of the hBD-2 genes. In general, poorly differentiated SCC has a more aggressive course and a worse prognosis than well-differentiated SCC. The inflammatory reactions induced by bacterial infection promote tumor cell growth with angiogenesis via formation of nitric oxide (NO) [8]. The aggressive behavior of poorly differentiated SCC may be related, among other mechanisms, to the susceptibility to bacteria caused by decreased hBD-2 expression.

In conclusion, the present study has documented the localization pattern of hBD-2 in oral epithelium and epithelial lesions. The results suggest that keratinization in oral epithelium plays an important role for the retention of the peptide in the epithelium and thus for its biological activity.

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