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Microscopic Anatomy

Periodontal study of ß-defensin 4 immunolocalization in the epithelium of human radicular cysts

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Abstract

Introduction

Periapical lesions are a host response that involves immune reaction to prevent dissemination of bacteria from an infected root canal. Radicular cysts are a sequela of granuloma formation characterized by the proliferation of the epithelial cell rests of Malassez and represents the most frequently encountered cyst of the jaws. Antimicrobial peptides are part of the innate local host response of multicellular organisms, such as plants, insects, amphibians, birds and mammals. They are microbicidal towards Gram-positive and Gram-negative bacteria, various pathogenic fungi including azole-resistant *Candida albicans* strains and even some enveloped viruses. The present work was undertaken to identify the expression of human β-defensin 4 in 10 radicular cysts, thus providing new insights into the development of periapical lesions.

Results

Cytoplasmic staining for human β-defensin 4 was detected in the epithelium of radicular cysts; therefore, detection of human β-defensin 4 suggests the involvement of innate immune response in radicular cyst formation.

Conclusion

These cysts play a protective role in preventing infection without expanding into adjacent tissues, such as alveolar bone. This study demonstrates that human β-defensin 4 may also contribute to the protective mechanism of radicular cysts.

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Introduction

Periapical lesions include periapical granulomas (PG) and radicular cysts (RC), and both are thought to represent different stages of the same inflammatory process[1,2]. These lesions are much more frequent than other jaw cysts[3]. Periapical disease represents the progression of a bacterial infection from the dental pulp to apical foramen that results in a localized inflammatory response concomitant with bone resorption. Periapical lesions are a host response that involves immune reaction to prevent dissemination of bacteria from an infected root canal. RCs are a sequela of granuloma formation characterized by the proliferation of the epithelial cell rests of Malassez and represent the most frequently encountered cysts of the jaws[4].

Although these lesions have been described histologically, very little is currently known about the precise mechanisms of development and growth inside the bone, and various molecular mechanisms have been considered[5–7]. Antimicrobial peptides (APs) are abundant and widely distributed effectors of the innate immune response that are able to kill microbes by destructing their cell membranes[8,9]. They are primarily expressed in epithelial tissues where they limit infections in the first hours after microbial colonization. Defensins are an important subfamily of APs. They are a complex group of 4-kD open-ended cysteine-rich, cationic peptides that are divided into α- and β-defensins based on the location and the connectivity of six conserved cysteine residues. β-defensins exhibit antimicrobial activity against oral microbes including periodontitis-related bacteria, Candida, and papilloma virus and have been demonstrated to be involved in various inflammatory processes[10–12]. On the basis of these observations, this study was undertaken to identify the expression of human β-defensin 4 (HBD-4) in RC, thus providing new insights into the development of periapical lesions.

Materials and methods

This work conforms to the values laid down in the Declaration of Helsinki (1964). The protocol of this study has been approved by the relevant ethical committee related to our institution in which it was performed. All subjects gave full informed consent to participate in this study.

Patients and tissue preparation

Ten patients who were diagnosed with RC based on clinical criteria (pain, swelling of gingivae around the apex of a tooth, X-ray, percussion, etc.) were included as subjects in the present study. Radiolucency (12–30 mm in size) was seen around the apex of the subject teeth in all patients.

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No systemic disease was observed in any patients, and antibiotics had not been taken during the previous 6 months. Written informed consent was obtained from all patients before collection of the samples. Patients were fully informed about the surgical procedure, post-operative care, follow-up examinations and alternative treatment options. Indications for apical surgery were based on the guidelines of the consensus report of the European Society of Endodontology[13]. Exclusion criteria were advanced periodontal disease, root fracture or post-perforation. After flap elevation and osteotomy, the periapical lesion and the root end were located. After root-end resection using a fissure bur, the resected root tip and the pathological tissue were curetted out. The intent was to collect the pathological tissue attached to the cut root tip. Tissue specimens were obtained from the archives of the Department of Dentistry of Catania University, Italy and the Laboratório De Patologia Cirúrgica Universidade Federal Da Bahia Faculdade De Odontologia. Surgical specimens were obtained as part of the apicoectomy procedure. Diagnosis was based on histopathological criteria, clinical history and radiographic appearance. The age of the patients ranged from 19 to 59 years (mean = 43 years).

Immunohistochemistry

After removal, the tissues were fixed immediately in 10% neutral-buffered formalin at room temperature (RT). They were embedded in paraffin wax, and 5-µm serial sections were collected on silane-coated glass slides. Immunohistochemical analysis was conducted as previously reported[14]. Sections were incubated for 30 min in 0.3% H_2O_2/methanol to quench endogenous peroxidase activity and then rinsed for 20 min with phosphate-buffered saline (PBS; Bio-Optica, Italy). The unmasking of antigenic sites was done, using a microwave oven (750 W), irradiating sections (5 min × 3) in capped polypropylene slide holders with citrate buffer (pH 6). Then, the sections were incubated with diluted mouse monoclonal HBD-4 antibody (Santa Cruz Biotechnology, Inc., Dallas, TX) (diluted 1:100 in PBS) overnight at 4°C. The secondary antibody, biotinylated mouse/anti-rabbit IgG was applied (for 30 min, at RT), followed by the avidin–biotin–peroxidase complex (Vector Elite Kit Abbott, Chicago, IL) for 30 min, at RT. The immunoreaction was visualized by incubating the sections for 4 min in a 0.1% 3,30-diaminobenzidine and 0.02% hydrogen peroxide solution (DAB substrate kit, Vector Laboratories, CA). The sections were lightly counterstained with Mayer's haematoxylin (Histolab Products AB, Goteborg, Sweden) mounted in GVA mount (Zymed, Laboratories Inc., San Francisco, CA) observed with an Axioplan Zeiss light microscope (Germany) and photographed with a digital camera (Canon, Japan).

Evaluation of immunohistochemistry

HBD-4 staining status was identified as either negative or positive. Positive staining was defined as the presence of brown chromogen detection within the cytoplasm. Stain intensity and the proportion of immunopositive cells were also assessed by light microscopy. According to previous studies[15], the intensity of staining (IS) was graded on a scale of 0–4, according to the following assessment: 0 = no detectable staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining and 4 = very strong staining. The percentage of HBD-4 immunopositive cells (extent score [ES]) was independently evaluated by three investigators (two anatomical morphologists and one surgical pathologist) and scored as a percentage of the final number of 100 cells in five categories: 0 = <5%; + = 5%–30%; ++ = 31%–50%; +++ = 51%–75% and ++++ = >75%. Counting was performed at ×200 magnification. The epithelium was assessed separately for the basal cell layer, the parabasal layer and the superficial layer. Positive control consists of tissue sections of trachea cartilage. For negative control testing, the sections of periapical lesions were treated with normal rabbit serum instead of with specific antibodies.

Statistical analysis

Statistical analysis data were analysed using GraphPad Prism software version 5.00 for Windows (GraphPad Software, San Diego, CA, http://www.graphpad.com). Significant differences ($p < 0.05$) between groups were determined using Fischer's exact test and the chi-square test. Cohen's kappa was applied to measure the agreement between the three observers and averaged over all three to evaluate overall agreement using the following grading: 0–0.2 (slight), 0.21–0.40 (fair), 0.41–0.60 (moderate), 0.61–0.80 (substantial) and 0.81–1.0 (almost perfect).

Results

Examination of haematoxylin-stained sections using light microscopy demonstrated a varied morphology of the periapical lesions. At light microscopic examination, the specimens variably showed the presence of epithelium, fibrous connective tissue with varying degrees of leukocytic infiltration and newly formed blood vessels. The 10 RCs' lumen was lined by non-keratinized stratified squamous epithelium, with an average thickness of four to six layers.

Cytoplasmic staining for HBD-4 was detected in the epithelium of RC (Figure 1). RC epithelial lining presented a strong immunoreaction (IS: 3) (Figure 2). Over 50% of epithelial lining cells in RC were immunolabelled by HBD-4 (ES: +++)(Figure 3). The epithelium of RC showed HBD-4 immunolabelled cells mainly in the suprabasal region, but patchy and discontinuous immunolabelling of the basal layer could

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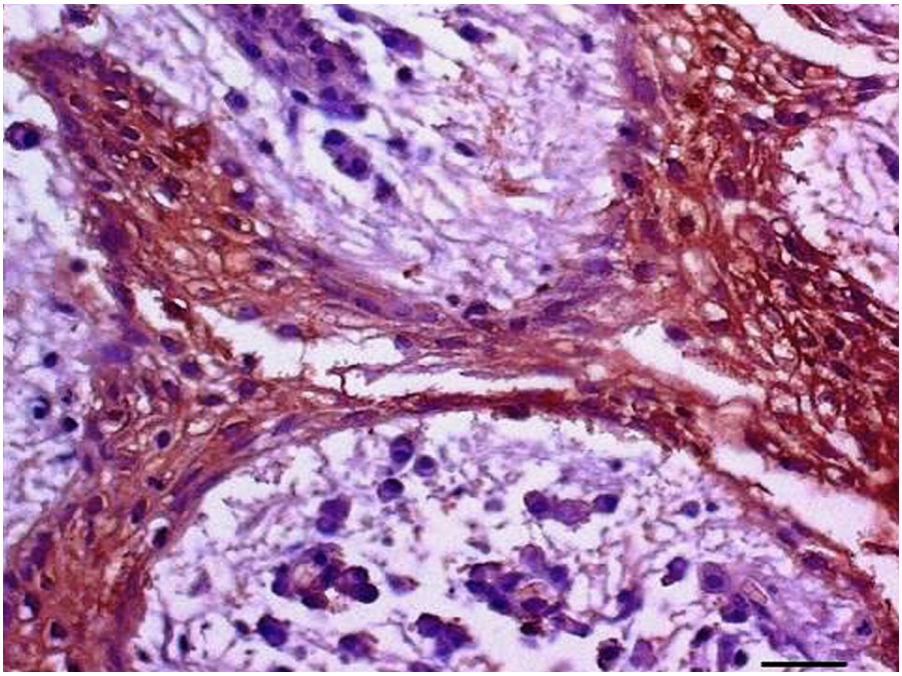

Figure 1: The non-keratinized stratified squamous epithelium of RC showed cytoplasmic strong staining for HBD-4. All epithelial layers were immunolabelled (×40, scale bar: 50 μm).

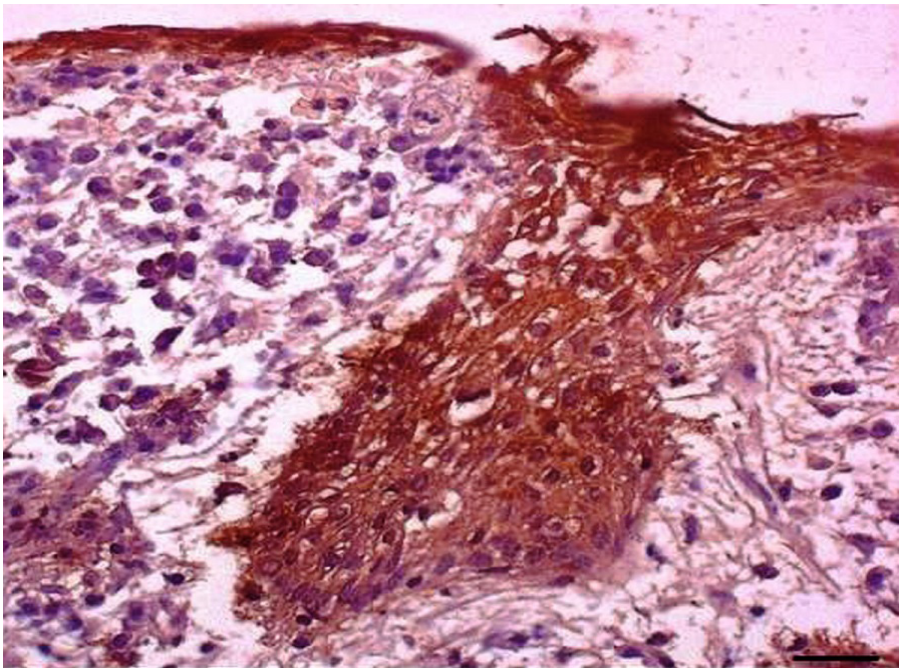

Figure 2: RC epithelial lining presented strong immunoreactions (×40, scale bar: 50 μm).

also be seen. Interobserver agreement, measured using the Cohen's kappa coefficient, was 0.94 (almost perfect). IS and ES scores are reported in Table 1.

Discussion

Apical periodontitis is primarily initiated and in most cases maintained by microorganisms living in the apical root canals of the affected teeth[16]. Some of these lesions contain epithelial cells that are believed to derive from the cell rests of Malassez. It is postulated that these cells serve as the source of the epithelium that lines the cavities of lesions that have developed into RC.

In humans, epithelial rests of Malassez are not proliferative, but previous studies indicate that proliferating epithelium is commonly found in inflammatory periapical lesions. Although the reasons for proliferation of epithelium in this disease are not clear, it is always connected with local accumulation of various types of immune cells[17]. The fact that PG with altered proliferating epithelium has larger number of immune cells than those of PG containing only the rests of Malassez correlates with this hypothesis[17].

APs are part of the innate local host response of multicellular organisms, such as plants, insects, amphibians, birds and mammals[18]. They are microbicidal towards Gram-positive and Gram-negative bacteria, various pathogenic fungi including azole-resistant *Candida albicans* strains[19] and even some enveloped viruses. Mammalian β-defensins have been isolated not only from neutrophils and other leukocytes, but also from epithelial cells, and from blood plasma and urine[20]. They are expressed predominantly in epithelial tissues, which provide the first line of defence between an organism and the environment[21]. The HBD-4 gene maps to chromosomal region 8p23 and encodes a prepropeptide of 72 amino acids. In human tissues, the highest level of HBD-4 expression was found in the testis and in the gastric antrum. Lower constitutive HBD-4 expression was observed in neutrophils and in the epithelia of the thyroid gland, the lung, the uterus and the kidney. Stimulation with heat-inactivated *Pseudomonas aeruginosa* or *Streptococcus pneumoniae* increased HBD-4 expression in human respiratory epithelial cells. HBD-4

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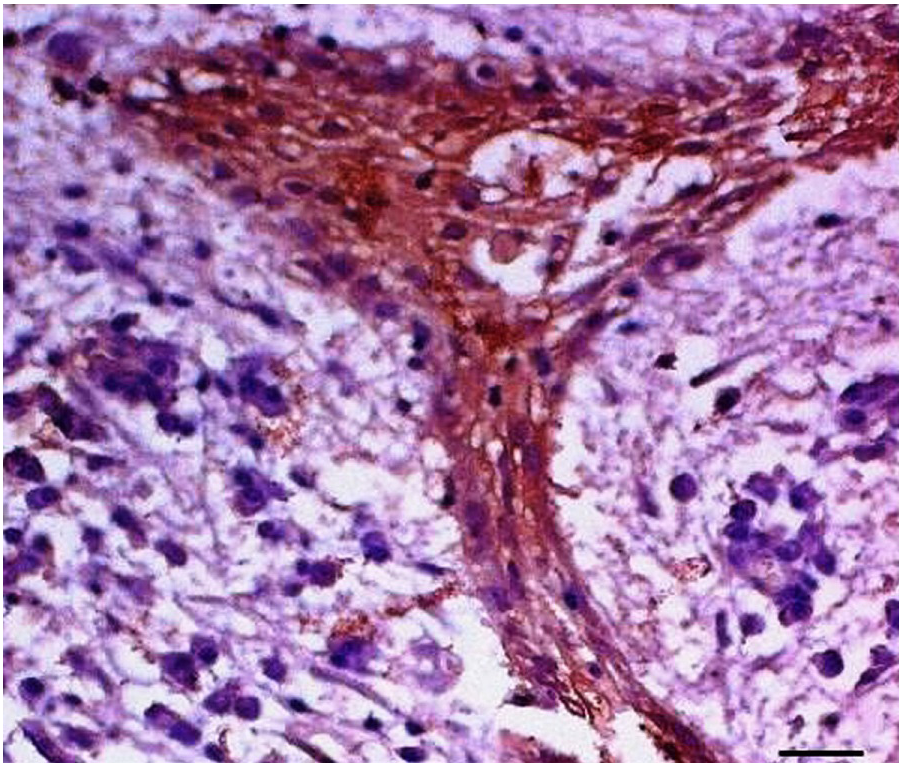

Figure 3: Over 50% of epithelial lining cells in RC were immunolabelled by HBD-4 (×40, scale bar: 50 μm).

Table 1 IS was graded on a scale of 0–4, according to the following assessment: 0 = no detectable staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining and 4 = very strong staining. ES scored as a percentage of the final number of 100 cells in five categories: 0 = <5%; + = 5%–30%; ++ = 31%–50%; +++ = 51%–75% and ++++ = >75%.

Intensity of staining	Extent score
Radicolar cysts	Radicolar cysts
IS value = 3	ES value = +++

displays weak antimicrobial activity against *Escherichia coli*, *Saccharomyces cerevisiae*, *Staphylococcus aureus*, *S. pneumoniae* and *Burkholderia cepacia*, and strong antimicrobial activity against *Staphylococcus carnosus* and *P. aeruginosa*[22]. Being positively charged, APs such as the defensins bind to negatively charged bacterial membrane targets. These include lipopolysaccharides in Gram-negative bacteria, polysaccharides and teichoic acids in Gram-positive bacteria and phospholipids (phosphatidylglycerol) in the inner membrane of both Gram-negative and Gram-positive bacteria. Besides their direct effects on microbial cells, there is a growing body of evidence that mammalian APs directly influence host cells, thereby inducing several mechanisms of the inflammatory processes and an immune response to the invading pathogens. Historically, the oral epithelium has been considered mainly as a passive covering that becomes damaged and ulcerated in disease. This view has changed dramatically and the epithelial compartment is now seen as providing both a physical barrier to infection and playing an active role in innate host defence[23]. Epithelial cells are in constant contact with bacterial products from supra- and sub-gingival biofilms on the tooth surface as well as from bacteria attached to mucosal surfaces. These cells respond to bacteria in an interactive manner; they secrete IL-8 and other chemokines and cytokines to alert various cell types and attract neutrophils. They also produce natural APs and proteins constitutively and inducibly in response to bacterial exposure. These APs are part of the innate immune system, a complex set of responses that keeps microbial invaders in check and maintains the microbial ecology of the healthy mucosa. Thus, the epithelium functions to actively respond to the environment, participates in response to infection, in signalling further host responses, and in integrating innate and acquired immune responses. β-defensin mRNA and peptides are expressed as a function of differentiation in cultured oral keratinocytes. In keratinocytes *in vitro*, the peptides are detected only in cells that are expressing involucrin, an early marker of differentiation[24]. In normal gingival tissue, mRNAs for both HBD-1 and HBD-2 are most strongly expressed in the spinous layer of the tissue, while the peptides are detected in the upper spinous, granular and cornified layers. The tissue location is consistent with a role for these peptides in the epithelial antimicrobial barrier. The lack of expression in the junctional epithelium, the suprabasal localization in stratified epithelia and the association with differentiation *in vitro*, all point to a dependence on normal differentiation for expression of β-defensins in stratified oral epithelia as well as in the epidermis[25].

Conclusion

This study is the first report to demonstrate the expression of HBD-4 in RC. Detection of HBD-4 suggests the

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involvement of innate immune response in RC formation. HBD-4 could induce proliferation and differentiation of epithelial rests of Malassez that evolve into the non-keratinized stratified squamous epithelium of RC. The results indicated that the epithelial rests of Malassez may actively participate in the inflammatory response to bacterial infection, and that they play an important role in the defence mechanism of the RC. The epithelial rests of Malassez are derived from the epithelium of Hertwig's epithelial root sheath during tooth root formation. Although a number of roles, such as endocrine function, and a protective or homeostatic role in the epithelial cells have been suggested[26], their essential role is still not clear. Their only confirmed activity is that in forming the epithelial lining of RC. RCs are formed as a result of the immunologic response to bacterial infection, followed by pulp tissue necrosis. These cysts play a protective role in preventing infection without expanding into adjacent tissue such as alveolar bone. This study demonstrates that HBD-4 may also contribute to the protective mechanism of RC.

References

1. García CC, Sempere FV, Diago MP, Bowen EM. The post-endodontic periapical lesion: histologic and etiopathogenic aspects. Med Oral Patol Oral Cir Bucal. 2007 Dec;12(8):585–90.
2. Leonardi R, Caltabiano M, Pagano M, Pezzuto V, Loreto C, Palestro G. Detection of vascular endothelial growth factor/vascular permeability factor in periapical lesions. J Endod. 2003 Mar; 29(3):180–3.
3. Leonardi R, Matthews JB, Loreto C, Musumeci G, Campisi G, Lo Muzio L, et al. Beta-catenin and surviving expression in Keratocystic Odontogenic Tumor (KCOT). A comparative immunohistochemical study in primary, recurrent and nevoid basal cell carcinoma syndrome (NBCCS)-associated lesions. Histol Histopathol (in press).
4. Nair PN. New perspectives on radicular cysts: do they heal? Int Endod J. 1998 May;31(3):155–60.
5. Leonardi R, Caltabiano R, Loreto C. Collagenase-3 (MMP-13) is expressed in periapical lesions: an immunohistochemical study. Int Endod J. 2005 May;38(5):297–301.
6. Leonardi R, Matthews JB, Caltabiano R, Greco M, Lombardo C, Loreto C, et al. MMP-13 expression in keratocyst odontogenic tumour associated with NBCCS and sporadic keratocysts. Oral Dis. 2010 Nov;16(8):795–800.
7. Loreto C, Galanti C, Leonardi R, Musumeci G, Pannone G, Palazzo G, et al. Possible role of apoptosis in the pathogenesis and clinical evolution of radicular cyst. An immunohistochemical study. Int Endod J. 2012 Nov (in press).
8. Zasloff M. Antimicrobial peptides of multicellular organisms. Nature. 2002 Jan;415(6870):389–95.
9. Habata I, Yasui T, Fujimori O, Tsukise A. Immunohistochemical aspects of antimicrobial properties in goat submandibular glands. Anat Histol Embryol. 2011 Feb;41(1):54–9.
10. Abiko Y, Saitoh M, Nishimura M, Yamazaki M, Sawamura D, Kaku T. Role of beta-defensins in oral epithelial health and disease. Med Mol Morphol. 2007 Dec;40(4):179–84.
11. Musumeci G, Carnazza ML, Leonardi R, Loreto C. Expression of β-defensin-4 in "an in vivo and ex vivo model" of human osteoarthritic knee meniscus. Knee Surg Sports Traumatol Arthrosc. 2012 Feb;20(2):216–22.
12. Sicurezza E, Loreto C, Musumeci G, Almeida LE, Rusu M, Grasso C, et al. Expression of β-defensin 4 on temporomandibular joint discs with anterior displacement without reduction. J Craniomaxillofac Surg (in press).
13. European Society of Endodontology. Quality guidelines for endodontic treatment: consensus report of the European Society of Endodontology. Int Endod J. 2006 Dec;39(12):921–30.
14. Musumeci G, Carnazza ML, Loreto C, Leonardi R, Loreto C. Defensin-4 (HBD-4) is expressed in chondrocytes derived from normal andosteoarthritic cartilage encapsulated in PEGDA scaffold. Acta Histoch. 2012 Dec;114(8):805–14.
15. Caltabiano R, Leonardi R, Musumeci G, Bartoloni G, Rusu MC, Almeida LE, et al. Apoptosis in temporomandibular joint disc with internal derangement involves mitochondrial-dependent pathways. An in vivo study. Acta Odontol Scand. 2013 May–Jul;71(3–4):577–83.
16. Nair PN, Sjögren U, Krey G, Kahnberg KE, Sundqvist G. Intraradicular bacteria and fungi in root-filled, asymptomatic human teeth with therapy-resistant periapical lesions: a longterm light and electron microscopic follow-up study. J Endod. 1990 Dec;16(12):580–8.
17. Matsuo T, Ebisu S, Shimabukuro Y, Ohtake T, Okada H. Quantitative analysis of immunocompetent cells in human periapical lesions: correlations with clinical findings of the involved teeth. J Endod. 1992 Oct;18(10):497–500.
18. Schröder JM. Epithelial antimicrobial peptides: innate local host response elements. Cell Mol Life Sci. 1999 Oct;56 (1-2):32–46.
19. De Lucca AJ, Walsh TJ. Antifungal peptides: novel therapeutic compounds against emerging pathogens. Antimicrob Agents Chemother. 1999 Jan;43(1):1–11.
20. Bensch KW, Raida M, Mägert HJ, Schulz-Knappe P, Forssmann WG. hBD-1: a novel β-defensin from human plasma. FEBS Lett. 1995 Jul;368(2):331–5.
21. Weinberg A, Krisanaprakornkit S, Dale BA. Epithelial antimicrobial peptides: review and significance for oral applications. Crit Rev Oral Biol Med. 1998;9(4):399–414.
22. Garcia JR, Krause A, Schulz S, Rodríguez-Jiménez FJ, Klüver E, Adermann K, et al. Human β-defensin 4: a novel inducible peptide with a specific salt-sensitive spectrum of antimicrobial activity. FASEB J. 2001 Aug;15(10): 1819–21.
23. Dale BA. Periodontal epithelium: a newly recognized role in health and disease. Periodontology. 2002;30:70–8.
24. Krisanaprakornkit S, Jotikasthira D, Dale BA. Intracellular calcium in signaling human β-defensin-2 expression in oral epithelial cells. J Dent Res. 2003 Nov;82(11):877–82.
25. Liu AY, Dstoumieux D, Wong AV, Park CH, Valore EV, Liu L, et al. Human β-defensin-2 production in keratinocytes is regulated by interleukin-1, bacteria, and the state of differentiation. J Invest Dermatol. 2002 Feb;118(2): 275–81.
26. Wessenlink PR, Beertsen W. The prevalence and distribution of rests of Malassez in the mouse molar and their possible role in repair and maintenance of the periodontal ligament. Arch Oral Biol. 1993 May;38(5):399–403.

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