

Induction of β -Defensin Expression by *Porphyromonas gingivalis*-Infected Human Gingival Graft Transplanted in *nu/nu* Mouse Subdermis

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It is important to understand the onset of periodontal disease in terms of bacterial infection and host factors. Host-bacteria interactions can be elicited in human cultured cells and animal models, but these models provide only limited biological information about human host reactions against bacterial attacks. Development of an *in vivo* model using human gingival tissue is needed. We established an *in vivo* model using *nu/nu* mice and evaluated host defense following bacterial infection in human gingiva. Human gingival samples were collected from periodontitis patients and transplanted in *nu/nu* mouse subdermis. After 2 weeks, human characteristics were confirmed by positive immunohistochemical reactions for human-specific markers. We used this model to investigate human β -defensin-2 (hBD-2), an antimicrobial peptide that contributes to initial defense against bacterial invasion. Using real-time polymerase chain reaction, *in situ* hybridization, and immunohistochemistry, we investigated whether hBD-2 expression was induced in human gingiva as a response to *Porphyromonas gingivalis* as a periodontal pathogen. Two hours after infection with bacteria, we detected increased expression of hBD-2 mRNA, which was localized in the epithelium of human gingiva. Using our *in vivo* model, we concluded that increased hBD-2 may play an important role in early defense from bacterial infection in human gingival epithelium.

Key words: human β -defensin-2 (hBD-2), human gingiva, *in vivo* model, periodontal disease, nude mice

I. Introduction

Periodontal diseases are highly prevalent and include gingivitis and chronic periodontitis [16]. Chronic periodontitis is caused by pathogenic oral bacteria such as *Porphyromonas gingivalis*. *P. gingivalis* is a Gram-negative, black-pigmented anaerobe that induces chronic inflammation in periodontal tissues such as the gingiva,

cementum, periodontal ligament, and alveolar bone [4, 15]. Gingival tissue of stratified squamous epithelium is directly attacked by bacteria. This tissue is not only a barrier but is also part of the innate immune system, because host responses also cause bacteria exclusion [6, 21]. Research into human host responses is important in the analysis of periodontal diseases [21]. So far, these biological responses have been evaluated in human cultured cells and various animal models [15, 24]. However, the information obtained from such *in vitro* models about the host response is insufficient. In addition, experiments using animals sometimes show differences in the reaction between humans and animals. To solve these problems, immunodeficient *nu/nu*

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mice or *scid/scid* mice have been widely used as *in vivo* models with human characteristics because various human tissues, including oral mucosa, can be transplanted into these mice [8, 10, 22, 31]. In oral mucosa, transplanted grafts frequently show cystic formation [10]. The native condition of human mucosal tissue was attenuated by this phenomenon. Tsukinoki *et al.* [27] established a transplantation technique for normal human oral mucosa that does not exhibit cystic changes using immunodeficient *nu/nu* mice, which were derived from a cross of *nu/nu* mice and *scid/scid* mice [9, 26]. However, the transplantation of gingival tissue from periodontal diseases has yet to be established in common immunodeficient *nu/nu* or *scid/scid* mice. The development of such a model will be useful for investigating relationships between host responses of gingival squamous epithelium and pathogen attacks.

Expression of antimicrobial peptides as a host response has been detected in all human epithelium, including oral epithelium, and is an important part of epithelial function [3]. These antimicrobial peptides have a broad spectrum of activity against both Gram-positive and Gram-negative bacteria as well as against yeast and viruses [3]. One type of antimicrobial peptide that plays an important role in host defense is defensins [1, 6]. Defensins are small, cationic antimicrobial peptides with a structure that contains disulfide bonds. They are classified into two subfamilies, α - and β -defensins, and the spectrum of antibacterial activity varies individually [6]. Among these defensins, human β -defensin 2 (hBD-2) is expressed in epithelium, including skin, lung, vagina, and oral mucosa, and exhibits potent antimicrobial activity against Gram-negative bacteria and fungi [1]. hBD-2 is typically produced by epithelial tissues after stimulation with microorganisms and proinflammatory mediators [4, 12], and contributes to initial defense in innate immune response. hBD-2 is expressed in the gingival epithelium in periodontal diseases in human biopsy samples (*in vivo*) and *in vitro* studies [2, 4, 24, 28]. However, the relationship between induction of hBD-2 in gingival epithelium and bacterial infection by *P. gingivalis* is not well known. In addition, recent data have demonstrated that hBD-2 is upregulated in the inflamed mucosa of patients with ulcerative colitis [30]. These studies indicated that hBD-2 is regulated by Toll-like receptor (TLR) 2 and TLR4 signaling in human intestinal epithelial cells.

The purpose of this study was to establish an *in vivo* experimental model for investigation of the host-bacteria interaction using conventional immunodeficient mice. We examined the expression profile of hBD-2 as a host response to *P. gingivalis* infection in human gingiva using this novel model. We also investigated whether TLR expression is associated with hBD-2 in gingival epithelium.

II. Materials and Methods

Animals

Six- to 8-week-old male BALB/c *scid/scid* and *nu/nu* mice, each weighing 20–25 g (CLEA Japan, Inc., Tokyo,

Japan), were used in this study. Mice were kept in a specific pathogen-free room and had free access to autoclaved food and sterile water.

Human tissue

After obtaining informed consent, gingival tissues were collected from 25 patients (10 men and 15 women; mean age, 58 ± 14.5 years) with chronic marginal periodontitis during periodontal surgery at Yokohama Clinic, Kanagawa Dental College (Yokohama, Japan). The Committee of Ethics of the Kanagawa Dental College approved the present study (Approval No. 104-20090803). Patients received preoperative initial preparation. Tissue was collected from the external marginal epithelium in all patients. Grafts from 8 cases were transplanted into *scid/scid* mice, and grafts from 17 cases were transplanted into *nu/nu* mice.

Transplantation methods

We modified the transplantation method of Tsukinoki *et al.* [27]. The collected tissue was immediately transferred to a mouse on a clean bench as follows. The tissues were immersed in tetracycline hydrochloride, and the size was adjusted to 3×2 mm before transplantation. In addition, subepithelial connective tissue was removed. Each mouse was anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and a flap was formed on the dorsal epidermis. The human gingival tissue was sutured into the flap so that the connective tissue side of the graft was placed onto the murine subepithelial tissue (Fig. 1). The flap was returned to its original position and sutured. The surgical area was covered with Tegaderm[®] (Sumitomo 3M, Ltd., Tokyo, Japan), and the mice were kept in a specific pathogen-free room for 2 weeks.

Bacterial conditions and infection methods

The bacterial strain used was *P. gingivalis*, which was obtained from the American Type Culture Collection 33277 (ATCC 33277; Manassas, VA, USA). *P. gingivalis* strains were grown at 37°C for 18 hr in brain heart infusion broth (BHI; Difco Laboratories, Sparks, MD, USA) containing 5 mg/ml yeast extract, 5 μ g/ml hemin, and 10 μ g/ml vitamin K₁ in an anaerobic chamber with an atmosphere of 85% N₂, 10% H₂, and 5% CO₂. In the present study, the transplanted gingival tissues were exposed to 7.1×10^8 cells/ml *P. gingivalis* ATCC 33277 in BHI 1.0 ml medium by subcutaneous injection for 2 hr (Fig. 1). Control transplanted grafts were treated with BHI medium. The experimental protocol used in this study was reviewed and approved by the Committee of Ethics on Animal Experiments of Kanagawa Dental College and was carried out in accordance with the Guidelines for Animal Experimentation of Kanagawa Dental College.

Histological and immunohistochemical analysis

Collected or transplanted gingival tissues were fixed in 4% paraformaldehyde for 24 hr and embedded in paraffin or OCT compound (Sakura Finetek Japan Co., Ltd., Tokyo,

Japan). To confirm morphological changes in gingival tissue, hematoxylin and eosin staining was performed according to standard routine techniques.

Subsequently, immunohistochemical analysis was performed using a Histofine staining kit (Nichirei Biosciences Inc., Tokyo, Japan), according to the instruction manual. To activate antigen for involucrin, Ki-67, and vimentin, slides were autoclaved at 121°C for 5 min in 10 mM citrate buffer (pH 6.0). Sections were then preincubated in 3% H₂O₂ for 15 min. After washing in phosphate-buffered saline, sections were incubated with anti-human involucrin polyclonal antibody (1:100; Harbor Bio-Products, Norwood, MA, USA), anti-human Ki-67 monoclonal antibody (MIB-1, 1:50; Dako Denmark A/S, Glostrup, Denmark), anti-human vimentin monoclonal antibody (Nichirei), or anti-human CD34 monoclonal antibody (Nichirei) for 1 hr at room temperature. To confirm that human-specific antibodies did not react with murine tissue, we also investigated immunoreactivity in normal murine skin.

To observe localization of hBD-2, TLR2, and TLR4 in the grafts, the tissue was embedded in OCT compound. Four-micrometer-thick frozen tissue sections were incu-

bated with anti-human hBD-2 polyclonal antibody (1:200; Peptide Institute, Inc., Osaka, Japan), anti-human TLR2 polyclonal antibody (TL2.1, 1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or anti-human TLR4 polyclonal antibody (H-80, 1:200; Santa Cruz Biotechnology) overnight at 4°C.

Subsequently, sections were treated with horseradish peroxidase-labeled secondary antibody (Nichirei) for 30 min at room temperature. The chromogen used was 3,3'-diaminobenzidine-tetrahydrochloride containing 0.003% H₂O₂ in phosphate-buffered saline. The sections were counterstained with hematoxylin or methyl blue. As a negative control, phosphate-buffered saline was used instead of primary antibody.

RNA isolation and real-time polymerase chain reaction (PCR)

Total RNA was isolated from gingival tissue using the ISOGEN reagent (Nippon Gene Co., Ltd., Toyama, Japan), according to the manufacturer's instructions. The quality of RNA was judged from the pattern of ribosomal RNA after electrophoresis through a 1.5% agarose gel containing ethidium bromide and visualization by UV illumination. RNA concentrations were determined with a NanoDrop 1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). cDNA was synthesized from total RNA using a first-strand cDNA synthesis kit (Roche Diagnostics Ltd., Lewes, UK) according to the manufacturer's instructions. Real-time PCR was performed using a LightCycler system (Roche) according to the manufacturer's instructions. The primer sequences used to amplify hBD-2 were 5'-TCT TCT CGT TCC TCT TCA TA-3' (forward) and 5'-TGT TTA TAC CTT CTA GGG CA-3' (reverse; PCR product: 127 bp) as designed and synthesized by Nihon Gene Research Laboratory (Sendai, Miyagi, Japan). Other target sequences were amplified using LightCycler Primer sets (Search-LC, Heidelberg, Germany). The PCR reactions were 95°C for 10 min followed by 35 cycles of 95°C for 10 sec, 58°C for 20 sec, and 72°C for 10 sec for hBD-2, and 95°C for 10 min followed by 35 cycles of 95°C for 10 sec, 68°C for 20 sec, and 72°C for 16 sec for TLR2 and TLR4. β-actin was used as a housekeeping gene (95°C for 10 min followed by 40 cycles of 95°C for 10 sec, 62°C for 10 sec, and 72°C for 10 sec). Gene expression is given as the ratio of the copy number of individual gene mRNA to β-actin mRNA for each sample.

In situ hybridization

Complementary RNA (cRNA) probes were produced by *in vitro* transcription of linearized pGEM-T Easy Vector (Promega Co., Madison, WI, USA). The two probes were chemically synthesized 120-mers of sense and antisense oligonucleotides specific for hBD-2 (nucleotides 56–175 and 217–336 of the hBD-2 coding sequence, Accession number: NM_004942). Digoxigenin (DIG)-11-UTP-labeled single-stranded cRNA probes for hBD-2 were prepared using a DIG labeling kit SP6/T7 (Roche) according to

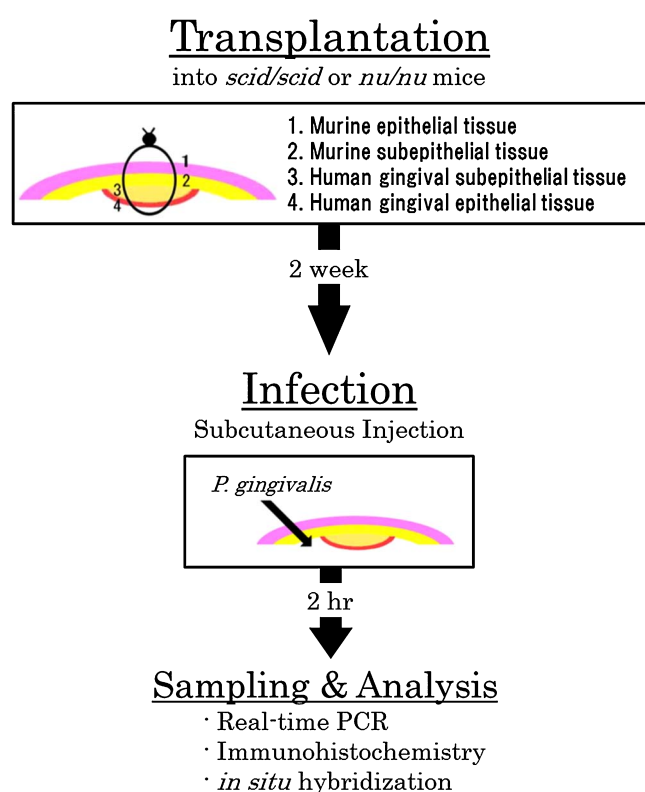


Fig. 1. Experimental designs used for investigating the host response against *P. gingivalis* in human gingiva. Schema for transplantation, infection experiments, and sampling. Human gingival samples were collected from periodontitis patients and transplanted into immunodeficient mice. After 2 weeks, the grafts were infected with *P. gingivalis* by subcutaneous injection in the mice. The grafts exposed to *P. gingivalis* for 2 hr were analyzed in mRNA and protein levels.

the manufacturer's instructions. Procedures for *in situ* hybridization were as described previously [11, 20]. Four-micrometer-thick paraffin sections were digested with 1 $\mu\text{g}/\text{ml}$ proteinase K for 20 min at 37°C. Hybridization was performed at 50°C for 17 hr using DIG-11-UTP-labeled single-stranded cRNA probes dissolved in hybridization medium (Wako Pure Chemical Industries, Ltd., Tokyo, Japan). After hybridization, mRNA was detected colorimetrically using a DIG-non-radioactive nucleic acid detection kit (Roche).

Statistical analysis

Statistical analyses were carried out using SPSS Version 17.0 (SPSS, Inc., Chicago, IL, USA) statistics programs. Mean mRNA levels were compared with the Mann-Whitney U test. All measurements are expressed as the group mean SEM. P values <0.05 were considered statistically significant.

III. Results

Transplantation success rate and histological findings of grafts

Parakeratosis, mild epithelial spike, and mild inflammatory cell infiltration were observed in the dissected gingival tissue before transplantation (Fig. 2A).

Transplantation into *scid/scid* mice was performed with tissue from 8 cases. All cases showed strong fibrosis with inflammatory cell infiltration (asterisk), focal abscess formation, and fragmentation of epithelium (Fig. 2B). The normal structures of human gingival tissue cannot be maintained by postoperative infection (0/8: 0%), further analysis was not performed.

In contrast, all grafts (17 cases) into *nu/nu* mice maintained normal structure, including the absence of cystic transformation and strong fibrosis in the epithelium (Fig. 2C, D). The unstimulated grafts transplanted into the

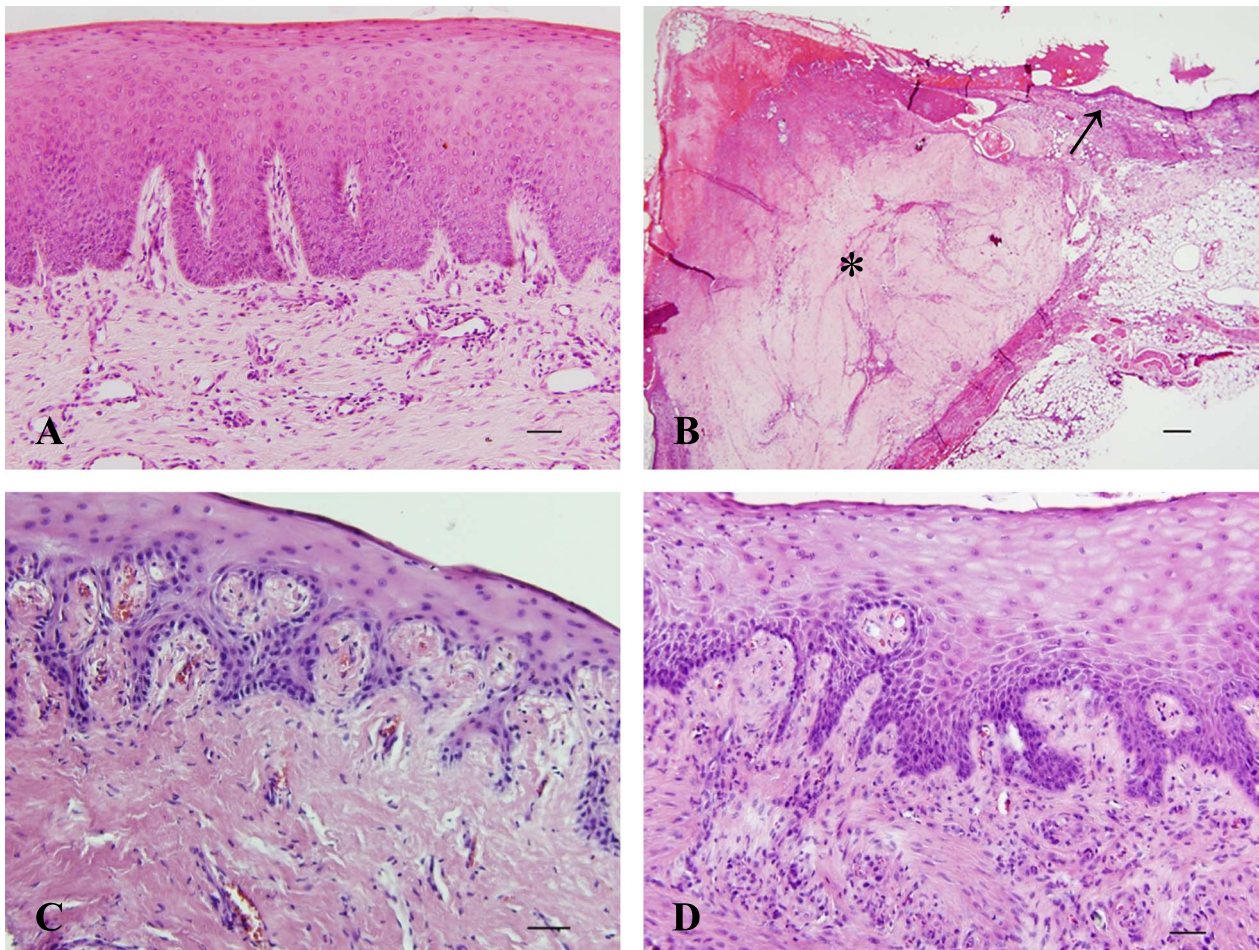


Fig. 2. Histological findings of human gingiva with hematoxylin and eosin staining. (A) Human gingival tissue showed parakeratosis, mild epithelial spike, and mild inflammatory cell infiltration before transplantation (Bar=50 μm). (B) Tissue grafted into *scid/scid* mice showed strong fibrosis with inflammatory cell infiltration (*), focal abscess formation, and fragmentation of the epithelium (arrow: murine skin epithelium, Bar=200 μm). (C, D) Tissue grafted into *nu/nu* mice showed retention of the basal layer in the epithelium and no changes in the connective tissue. The unstimulated grafts showed little inflammatory cell infiltration (C, Bar=50 μm), whereas stimulated grafts showed marked infiltration of inflammatory cells in subepithelial tissue and mild intraepithelial infiltration (D, Bar=50 μm).

mice showed little inflammatory cell infiltration, and the presence of bacteria was not noted (Fig. 2C). We observed a tendency towards a decrease in inflammatory cell infiltration into the grafts compared with before transplantation. Transplanted grafts stimulated with *P. gingivalis* showed marked infiltration of inflammatory cells in subepithelial tissues and mild intraepithelial infiltration (Fig. 2D). Moreover, the basal layer of the epithelium was maintained and the connective tissue exhibited no abnormal arrangement or thickness of collagen fibers. These histological findings in transplanted grafts into *nu/nu* mice showed maintenance of the normal structure of epithelial tissue (17/17, 100%) and subepithelial connective tissue, similar to those before transplantation. These findings suggest that *nu/nu* mice are suitable for transplantation rather than *scid/scid* mice. Therefore, the experimental data shown are from *nu/nu* mice.

Immunohistochemical profiles in human gingiva

We next determined immunohistochemically whether

transplanted grafts maintained human characteristics after 2 weeks using human-specific markers in the *nu/nu* mice. Involucrin, a differentiation marker, showed a widely positive reaction in the epithelium, but was not observed in the subepithelial connective tissues (Fig. 3A). Ki-67, a proliferation marker, was localized within many cells of the basal layer (Fig. 3B). Vimentin, which forms the cytoskeleton in nonepithelial cells, was detected in fibroblasts and endothelial cells of the subepithelial connective tissue (Fig. 3C). CD34, a marker of endothelial cells, was observed in endothelial cells (Fig. 3D). These immunohistochemical patterns were consistent with expression patterns in original gingival tissues before transplantation (data not shown). No apparent immunoreactivity was observed in normal murine skin using human-specific antibodies (data not shown).

Expression of hBD-2, TLR2, and TLR4 after *P. gingivalis* stimulation

We used real-time PCR to analyze levels of hBD-2,

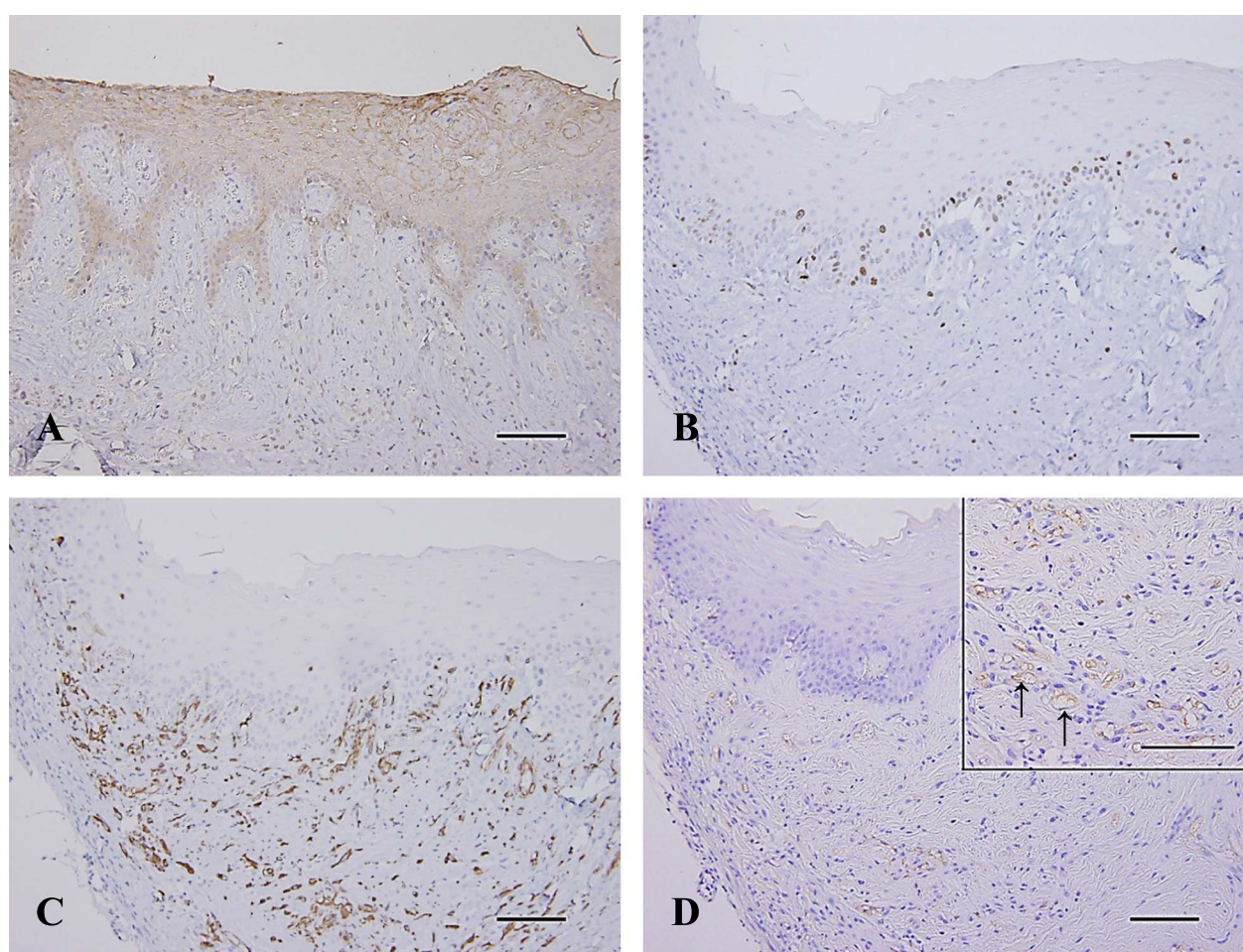


Fig. 3. Human-specific markers specifically expressed in the gingival grafts transplanted into *nu/nu* mice. Immunohistochemical analysis with human-specific primary antibodies. (A) Involucrin was widely expressed in the epithelial tissue. (B) Ki-67-positive cells were detected in the basal layers of the epithelium. (C) Vimentin was detected in fibroblasts and endothelial cells of the subepithelial connective tissue. (D) CD34 expression was observed in endothelial cells (arrows). Bar=100 μ m.

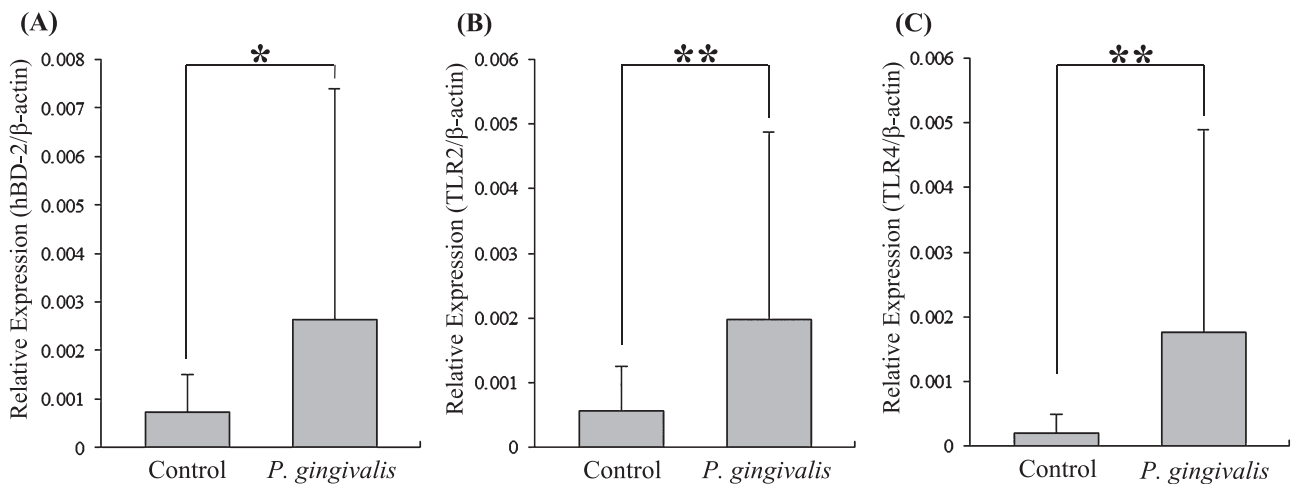


Fig. 4. Induction of hBD-2 (A), TLR2 (B), and TLR4 (C) mRNA in human gingiva. The expression levels of hBD-2, TLR2, and TLR4 in the transplanted gingival tissue were assayed using real-time PCR. Data are the ratio of each mRNA level to β -actin. Control: unstimulated, *P. gingivalis*: stimulated with *P. gingivalis* for 2 hr. Values are the mean \pm SEM; $n=17$ samples in each group. * $p<0.05$, ** $p<0.01$.

TLR2, and TLR4 mRNA in human gingival tissue and statistically compared differences in mRNA expression following *P. gingivalis* stimulation compared to the control group. hBD-2 expression levels increased significantly ($p<0.05$) in the stimulation group (0.0026 ± 0.0047) compared with the control (0.0007 ± 0.0008 , Fig. 4A). Moreover, *P. gingivalis* stimulation induced significant upregulation of TLR2 mRNA (stimulation group, 0.0020 ± 0.0029 ; control group, 0.0006 ± 0.0007 ; $p<0.01$, Fig. 4B) and TLR4 mRNA (stimulation group, 0.0018 ± 0.0031 ; control group, 0.0002 ± 0.0003 ; $p<0.01$, Fig. 4C).

hBD-2 in situ hybridization

hBD-2 mRNA signals were not detected in the cytoplasm of unstimulated grafts transplanted into mice, but weak non-specific signals were observed in the nucleus of sections reacted with antisense probes (Fig. 5A). In transplanted grafts stimulated with *P. gingivalis*, positive hBD-2 mRNA signals were noted in the cytoplasm, and weak, non-specific signals were seen in the nucleus. These signals were mainly localized in the basal and suprabasal layers of the grafts (Fig. 5B). Sense probes showed no hybridization signals in unstimulated (Fig. 5C) or stimulated grafts (Fig. 5D). Stimulated grafts showed increased background signal in the nucleus.

hBD-2, TLR2, and TLR4 immunohistochemistry in human gingiva

Immunohistochemical analysis showed expression of hBD-2, TLR2, and TLR4 proteins in the transplanted grafts (Fig. 6). In the unstimulated condition, no apparent expression of hBD-2 was detected in transplanted human gingival tissue (Fig. 6A). Stimulated grafts (Fig. 6B) showed marked expression of hBD-2 in spinal layers of the epithelium compared with the control group. hBD-2 exhibited not only cell membrane localization but also intracellular staining.

No expression was observed in connective tissue. In the unstimulated condition, no expression of TLR2 (Fig. 6C) or TLR4 (Fig. 6E) was detected. However, TLR2 (Fig. 6D) and TLR4 (Fig. 6E) were observed in the spinal layers in stimulated grafts. Both molecules were primarily localized at the cell membrane.

IV. Discussion

Transplantation into *scid/scid* mice was unsuccessful. Because *scid/scid* mice have bushy hair but *nu/nu* mice are hairless, *scid/scid* mice may tend to experience post-operative infection. *scid/scid* mice are also more severely immunodeficient than *nu/nu* mice [22, 25]. In addition, *scid/scid* mouse skin is thinner and less elastic than skin of *nu/nu* mice. Gingival grafts with inflammation showed fibrosis of various degrees, although gingival grafts were curettaged to remove the subepithelial connective tissue during pretreatment before transplantation. The characteristics of the mice and the condition of the grafts are unfavorable for reconstruction of blood vessels. Re-establishing blood flow is important for graft stability as well as avoiding infection and preventing necrosis. *nu/nu* mice were considered to have characteristics that were well-suited for transplantation of gingival tissue compared to *scid/scid* mice.

From histopathological findings of grafts, polarity and cell morphology were preserved. More importantly, because the grafts showed little inflammatory infiltration, including neutrophils, in the unstimulated control groups after 2 weeks, the transplanted grafts may become infection-free in the subcutaneous layer in mice. This phenomenon indicated that transplanted gingival tissue attained near-normal conditions at the level of inflammation. In addition, the subcutaneous space is an anaerobic environment and is suited to infection with anaerobic bacteria such as *P. gingivalis*.

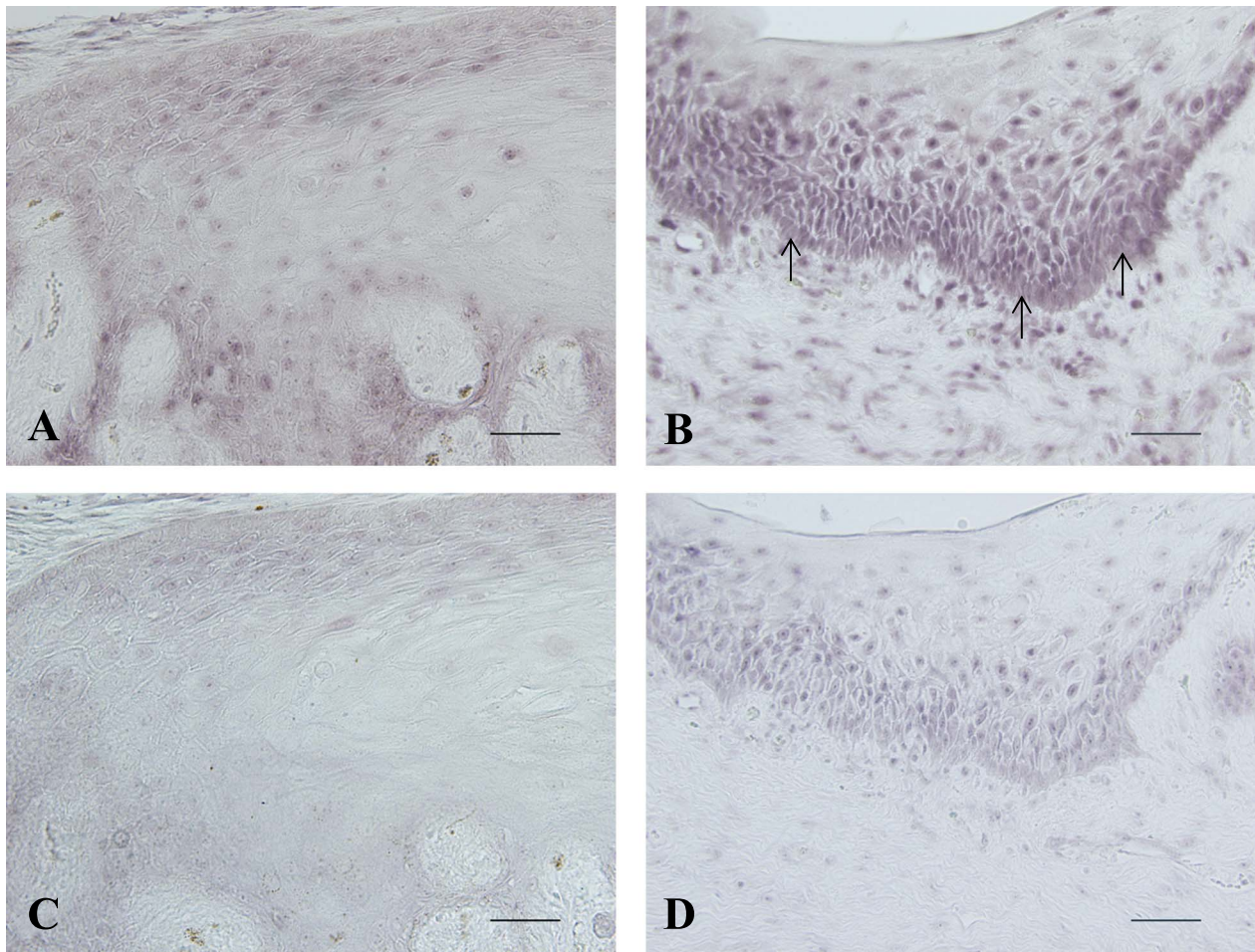


Fig. 5. Expression and localization of hBD-2 mRNA in human gingiva. Representative photomicrographs show the expression and localization of hBD-2 mRNA. The gingival tissues transplanted into mice were unstimulated (**A, C**) or stimulated with *P. gingivalis* (**B, D**). A high level of hBD-2 mRNA was detected in tissue stimulated with *P. gingivalis* (arrows, **B**) in sections treated with antisense probes compared to unstimulated tissue (**A**). Sense probes showed no hybridization signals in the grafts (**C, D**). Weak, non-specific signals are seen in the nucleus in sections reacted with sense and antisense probes. Bar=50 μ m.

Concomitantly, this environment is consistent with the gingival sulcus or periodontal pocket, which forms to accompany the destruction of periodontal tissue [16]. These observations suggest that this model adequately represents the situation of human periodontal tissue.

When human-specific antibodies were used to label epithelial and subepithelial connective tissue after 2 weeks, positive reactions were seen in transplanted grafts that were consistent with gingival tissue before transplantation. Thus, the transplanted grafts preserved human rather than mouse characteristics. Tsukinoki *et al.* [27] found that grafts in *nu/scid* mice maintained morphological and differentiation characteristics. The authors did not detect expression of CD34 in *nu/scid* mice [27], but CD34 expression was noted in endothelial cells of grafts into *nu/nu* mice. In the present study, endothelial cells may not have entirely converted to mouse characteristics, because the time that had elapsed after transplantation was shorter than that in the Tsukinoki *et al.* study. These data suggest that this model is useful for

analysis of native responses of gingival tissue to anaerobic bacteria, because grafts transplanted into *nu/nu* mice showed preservation of human characteristics, reproduction of the anaerobic environment, and decreased inflammation.

A significant difference in the expression of hBD-2 mRNA between transplanted grafts stimulated with live *P. gingivalis* and unstimulated grafts was detected at 2 hr with real-time PCR. Low expression of hBD-2 mRNA was found in unstimulated grafts. Taguchi *et al.* [24] reported that in cultured gingival epithelial cells, expression of hBD-2 is significantly enhanced by live *P. gingivalis* after 48 hr or 72 hr but not after 1, 3, 6, or 24 hr. In addition, after 72 hr, hBD-2 expression levels tended to be increased more than after 48 hr. The time points of 48 and 72 hr are generally sufficient to induce acquired immunity after infection. Although hBD-2 is induced by various bacterial infections, it is reasonable to show the timing of hBD-2 expression prior to induction of acquired immunity based on the unique characteristics of hBD-2. The cell culture conditions

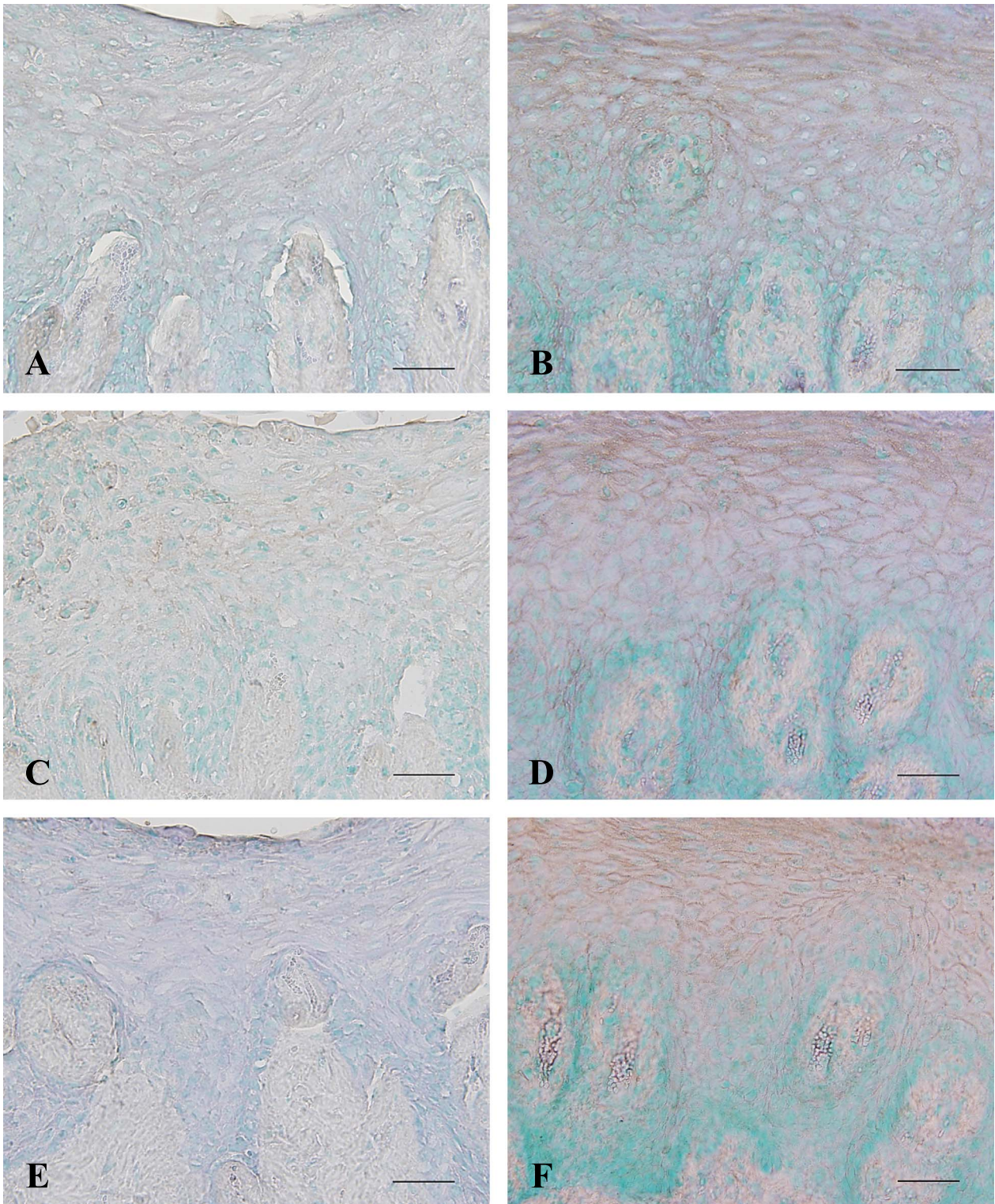


Fig. 6. Immunohistochemical localization of hBD-2, TLR2, and TLR4 in human gingiva. Representative photomicrographs show the immunohistochemical localization of hBD-2 (A, B), TLR2 (C, D), and TLR4 (E, F) protein. The transplanted gingival tissues were unstimulated (A, C, and E) or stimulated with *P. gingivalis* (B, D, and F). hBD-2 was detected in the spinal layers of the epithelium in stimulated grafts. hBD-2 exhibited cell membrane and intracellular distribution. TLR2 and TLR4 were localized at the cell membrane in the spinal layers. Bar=50 μ m.

reported by Taguchi *et al.* may lead to increased basal levels of hBD-2 expression in the control group, which showed hBD-2 expression beginning at 3 hr [24]. In contrast, our novel *in vivo* experimental model showed that induction of hBD-2 occurred in the early stages following live *P. gingivalis* infection. Thus, hBD-2 may play a role in the initial defense system in gingival epithelial cells following bacterial infection.

Domisch *et al.* [7] reported no statistically significant difference among healthy control, gingivitis, and chronic periodontitis groups in terms of hBD-2 expression in gingival tissue samples. Moreover, Vardar-Sengul *et al.* [28] reported that hBD-2 is significantly lower in the gingivitis group, but higher in the chronic periodontitis group relative to healthy controls. Thus, expression levels of hBD-2 in gingival specimens with periodontal diseases remain controversial. Interestingly, gingival epithelium synthesizes hBD-2, even in the healthy state [2, 7]. Therefore, basal levels of hBD-2 may be high in control healthy gingival epithelium. However, our results clearly demonstrated that hBD-2 levels in stimulated grafts were increased by *P. gingivalis* infection compared with control grafts. In patients with periodontitis that was caused by *P. gingivalis*, expression of hBD-2 may be higher than in non-inflamed gingival tissue.

We also found that hBD-2 mRNA was detected in the suprabasal layers, whereas the protein was localized in spinal layers of gingival epithelium. Dale *et al.* [5] reported that the distribution between mRNA and protein differs in human gingival epithelium. More importantly, we found that hBD-2 mRNA and protein were higher in the grafts stimulated with *P. gingivalis* than in unstimulated grafts. Thus, the expression of hBD-2 mRNA and protein may be induced by bacterial infection in human gingival epithelium.

TLRs recognize specific pathogen-associated molecular patterns [13, 17, 29] and play an important role in the induction of proinflammatory cytokines and antimicrobial peptides, which trigger innate immunity and acquired immunity [14, 18, 23]. In our study, the expression of TLR2 and TLR4 mRNA was upregulated in stimulated grafts. Previously, an *in vitro* study demonstrated that TLR signaling is related to induced expression of hBD-2 [13, 14, 19]. To determine whether this interaction occurs *in vivo*, we investigated changes in TLR2 and TLR4 expression following bacterial stimulation. The induction of TLR we observed was similar to that reported in past studies [17, 19, 23, 29], and the immunohistochemical localization was also consistent with that of hBD-2. Therefore, TLR may be involved in induced expression of hBD-2 following bacterial infection. Collectively, our results show that hBD-2 expression is actively induced in response to bacterial infection and TLR may regulate hBD-2 expression in human gingival epithelium.

In conclusion, to confirm associations between human gingival responses and bacterial infections, we established a novel *in vivo* experimental model using *nu/nu* mice.

This model demonstrated that expression of hBD-2 was induced soon after infections and was increased in gingival epithelium that was infected with *P. gingivalis*. hBD-2 expression in gingival epithelium may play an important role in protection against *P. gingivalis*. Further examination of TLR signaling as a possible mechanism of induction of expression of hBD-2 will be necessary in the future.

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VI. References

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