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Expression of inflammatory cytokines and beta-defensin 1 mRNAs in porcine epithelial rests of Malassez in vitro

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Abstract In the present study, we investigated the mRNA expression of inflammatory cytokines, including interleukin (IL)-1α, IL-6, IL-8, and granulocyte macrophage colony-stimulating factor (GM-CSF), and β defensin 1 (BD-1), an antimicrobial peptide, in the epithelial rests of Malassez in vitro. A reverse transcription-polymerase chain reaction (RT-PCR) assay was performed in order to observe the expression of these mRNAs. The effect of lipopolysaccharide (LPS) on the mRNA expression was also studied by quantitative RT-PCR assay, with a LightCycler, using the double-stranded DNA dye SYBR Green I. The mRNAs of the four kinds of inflammatory cytokines and BD-1 were detected in the epithelial cells under normal culture conditions. Immunocytochemical staining showed the expression of CD14, a receptor for LPS, on the epithelial cells. The mRNA expressions of IL-1α, IL-6, IL-8, and GM-CSF were upregulated by stimulation with LPS, in a dose- and time-dependent manner. Epithelial cells incubated with 1000 ng/ml of LPS for 6 h showed the most significant upregulation of the cytokine mRNAs. On the other hand, no obvious alteration of BD-1 expression by LPS stimulation was observed. 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 defense mechanism of the radicular cyst.

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Introduction

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,[1–3] their essential role is still not clear. Their only confirmed activity is that in forming the epithelial lining of radicular cysts. Radicular cysts 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. Bacterial infections often induce cytokine production in the adjacent tissue, causing an inflammatory response. The expression of inflammatory cytokines, including interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor-alpha (TNF-α) has been demonstrated in radicular cysts.[4–7] Recently, these cytokine mRNAs were detected in the lining epithelium, as well as in the connective tissue, of radicular cysts.[7] Although some types of epithelial cell produce certain kinds of inflammatory cytokines,[8–10] it is still unclear whether the epithelial rests of Malassez, which are the origin of the lining epithelium, express these cytokines. The present study investigated the mRNA expression of inflammatory cytokines, including IL-1α, IL-6, IL-8, and granulocyte macrophage colony-stimulating factor (GM-CSF) produced by epithelial rests of Malassez in vitro. Antimicrobial peptides may also contribute to the protective mechanism of radicular cysts. Defensin is a major antimicrobial peptide, and is classified into two classes, the α- and β-defensins. The α-defensins are mainly expressed in neutrophils, whereas β-defensins are mainly expressed in epithelial cells.[11–14] Therefore, the expression of β-defensin 1(BD-1) in the epithelial rests of Malassez was also investigated. The expression levels of the inflammatory cytokines and BD-1 were compared with and

without the stimulation of lipopolysaccharide (LPS), a bacterial endotoxin.

Materials and methods

Cell isolation and cell culture

Epithelial cells from porcine epithelial rests of Malassez were isolated from the periodontal ligament and cultured by a previously described method.[15,16] Briefly, explants of periodontal ligament obtained from an extracted pig premolar were cultured in Dulbecco modified Eagle medium (DMEM) (Sigma-Aldrich, UK) containing 10% fetal bovine serum (FBS; Cancera, Canada) and antibiotics (penicillin G potassium, 100 μg/ml [Meijiseika, Tokyo, Japan] and Fungizone, 30 μg/ml [Bristol-Myers Squibb Tokyo, Japan] at 37°C in a humidified atmosphere of 95% air and 5% CO2. An outgrowth, comprised of both epithelium-like and fibroblast-like cells, was observed after 2 or 3 weeks of incubation. The two cell types were separated into epithelial populations that were more and less resistant to detachment with 10% dispase (Goudousyusei, Tokyo, Japan). The cells that were less resistant were detached, removed from the rest of the cell population, and discarded. The attached cells were cultured. The separation procedure was repeated two or three times so as to remove any fibroblasts.

LPS stimulation

LPS derived from *Escherichia coli* (Sigma Chemical, St. Louis, MO, USA) was used for endotoxic bacterial stimulation. Epithelial cells were incubated with culture medium containing four different concentrations of LPS: 0 ng/ml (control), 10 ng/ml, 100 ng/ml, and 1000 ng/ml. Also, we selected three different incubation times: 1 h, 6 h, and 24 h.

Immunofluorescence method

The expression of CD14 in the epithelial cells was confirmed by an immunofluorescence method. The epithelial cells were cultured in a glass slide chamber (Nunc, Naperville, IL, USA) at a cell number of 5×10^5/ml, in DMEM. The slides were fixed in acetone at 4°C for 15 min. Samples were washed in distilled water and soaked in phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA; Sigma-Aldrich Chemie, Germany) to block nonspecific reaction. Samples were incubated at 4°C overnight with a monoclonal primary antibody at a 1:25 dilution (CD14, UCH-M1; Santa Cruz Biotechnology Santa Cruz, CA, USA). After the samples had been rinsed in PBS, a goat anti-mouse IgG antibody (Alexa Fluor 488; Molecular Probes, Eugene, OR, USA) was applied as the secondary antibody, at a 1:100 dilution, at room temperature for 1 h. The samples were rinsed with PBS and mounted (Biomeda, Foster City, CA, USA). As negative control, 2% BSA was used instead of the secondary antibody in the same procedure.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was performed on epithelial cells that had and had not been stimulated with LPS. Total RNA was extracted by the acid guanidium thiocyanate/ phenol-chloroform method, using a total RNA isolation reagent (Trizol Reagent, GIBCO BRL, MD, USA). The RNAs were reverse transcribed to cDNA, using Oligo(dT)$^{12-18}$ primer (GIBCO BRL), according to the manufacturer's instructions. The RT products were amplified using a PCR kit (AmpliTag Gold with GendAmpR 10× PCR Buffer [PerkinElmer, Applied Biosystem, Foster City, CA, USA]) and a thermal cycler (Takara PCR Thermal Cycler MP, Osaka, Japan) according to the PCR protocol (Clontech, Palo Alto, CA, USA). The PCR conditions were 45 s at 94°C, 45 s at 54°C, and 2 min at 72°C. The PCR products were separated on 2% agarose/ethidium bromide gels.

Quantitative RT-PCR with LightCycler

A quantitative RT-PCR assay was carried out with a LightCycler, using the double-stranded DNA dye SYBR Green I (Roche Diagnostics, Germany) in order to observe the level of mRNAs. The primers for the assay with the LightCycler were the same as those used in ordinary RT-PCR. Quantification was performed by comparison of standardized samples. In the present study, the concentration of cDNA in unstimulated samples was 1.5, 1, and 0.5 μl in each sample. The PCR conditions in the LightCycler are shown in Table 1. Melting curve analysis was also performed after the PCR amplification, to confirm that there was no primer dimer in the PCR products.

Results

In the epithelial cells, the mRNAs of the four kinds of inflammatory cytokines and BD-1 were detected by RT-PCR (Fig. 1). We confirmed the expression of CD14, a receptor for LPS, in the epithelial cells before they were stimulated with LPS. Immunostaining for CD14 was clearly observed in the epithelial cells (Fig. 2a). The reactions were mainly around the nuclei, and some reactions, observed as fibrillar structures, were also found at sites of cell-cell interaction and at the periphery of a single cell (Fig. 2b).

The expression levels of the cytokine and BD-1 mRNAs on stimulation with LPS were estimated by a semi-quantitative RT-PCR with a thermal cycler and by a quantitative RT-PCR with the LightCycler. The expression of IL-1α, IL-6, IL-8, and GM-CSF mRNAs was obviously upregulated by LPS stimulation in a dose- and time-dependent manner (Fig. 3a–d; Figs. 4, 5). The epithelial cells incubated with 1000 ng/ml of LPS for 6 h showed the most

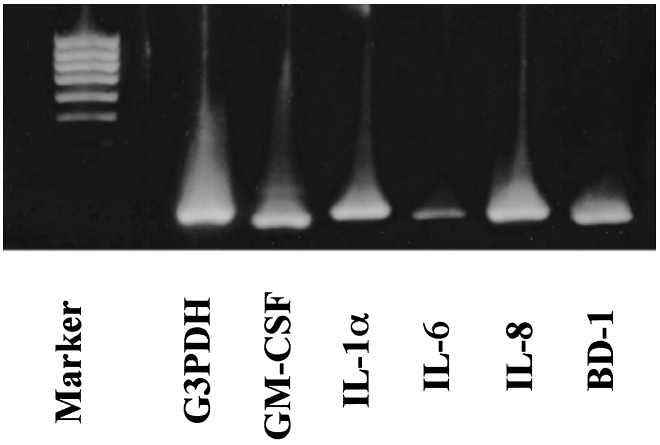

Fig. 1. Expression of mRNAs by epithelial cells without lipopolysaccharide (LPS) stimulation; 45 cycles. *From left to right*, Marker, *G3PDH*, granulocyte macrophage colony-stimulating factor (*GM-CSF*), interleukin I-α (*IL-1α*), IL-6, IL-8, and β-defensin-1 (*BD-1*). The mRNAs of the four kinds of inflammatory cytokines and BD-1 were detected by reverse transcriptase (RT)-polymerase chain reaction (PCR)

significant upregulation of the cytokine mRNAs (Fig. 3a–d). Under this condition, there was a 9.4-fold, a 4.2-fold, a 7.5-fold and a 23.8-fold increase as compared with expression in the control groups for GM-CSF, IL-1α, IL-6, and IL-8 mRNA expression, respectively (Figs. 4, 5). On the other hand, no obvious alteration of BD-1 expression by LPS stimulation was observed (Fig. 3e).

Discussion

The present study demonstrated, for the first time, that epithelial rests of Malassez in vitro expressed inflammatory cytokines, including IL-1, IL-6, IL-8, and GM-CSF, and BD-1 mRNAs. Also the upregulation of inflammatory cytokine expression by LPS stimulation was shown. Epithelial rests of Malassez form the lining epithelium of radicular cysts. Only a few studies have been reported concerning inflammatory cytokines in radicular cysts.[5,7] The lining epithelia of radicular cysts were reported to have expressed IL-

Table 1. Sequences of primers and annealing temperatures in LightCycler

Primer	Sequence	Size	Annealing temperature
G3PDH	Up: gga ctc atg acc acg gtc cat Down: tca gat cca caa ccg aca cgt	201 bp	64°C
GM-CSF	Up: tga cac agc ggc tgt gat gaa t Down: cac agg aag ttt cct cgg tga	199 bp	64°C
IL-1α	Up: gaa tac agt tct gat atc gac c Down: act gat tta aac tca acc gtc	213 bp	54°C
IL-6	Up: agc tat gaa ctc cct ctc cac a Down: cag aga ttt tgc cga gga tgt	199 bp	59°C
IL-8	Up: ctg cag ctc tct gtg agg ctg c Down: tcc ttg ggg tcc agg cag acc	198 bp	60°C
BD-1	Up: atg aga ctc cac cgc ctc ctc ct Down: gca gca ttt gac ttg ggg cat g	183 bp	64°C

G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; BD-1, β defensin-1

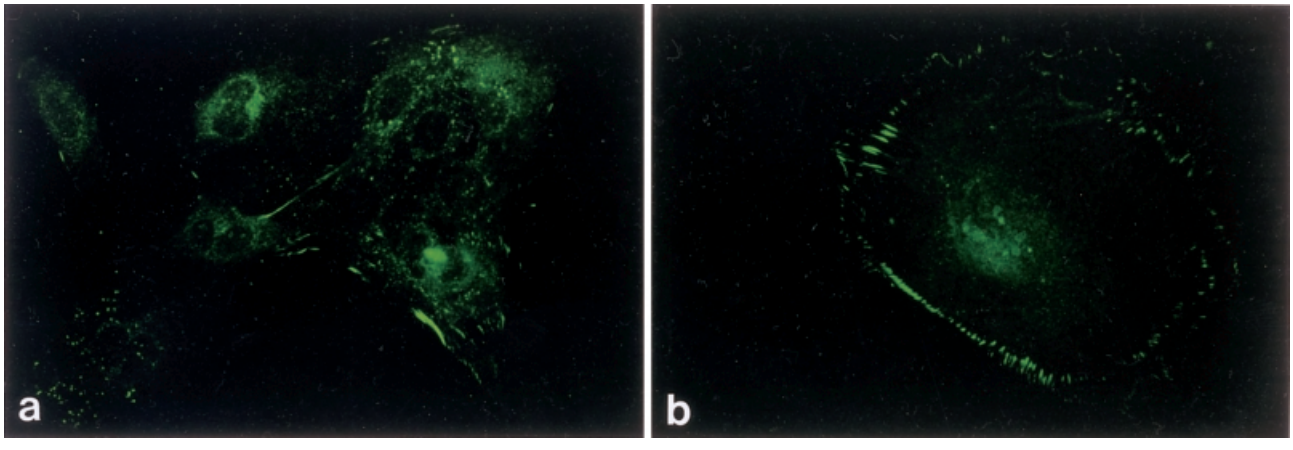

Fig. 2a,b. Immunofluorescence for CD14 on epithelial rests of Malassez. Expression of CD14 was clearly observed in the epithelial cells (**a**). The reactions were observed as fibrillar structures at the periphery of a single cell (**b**)

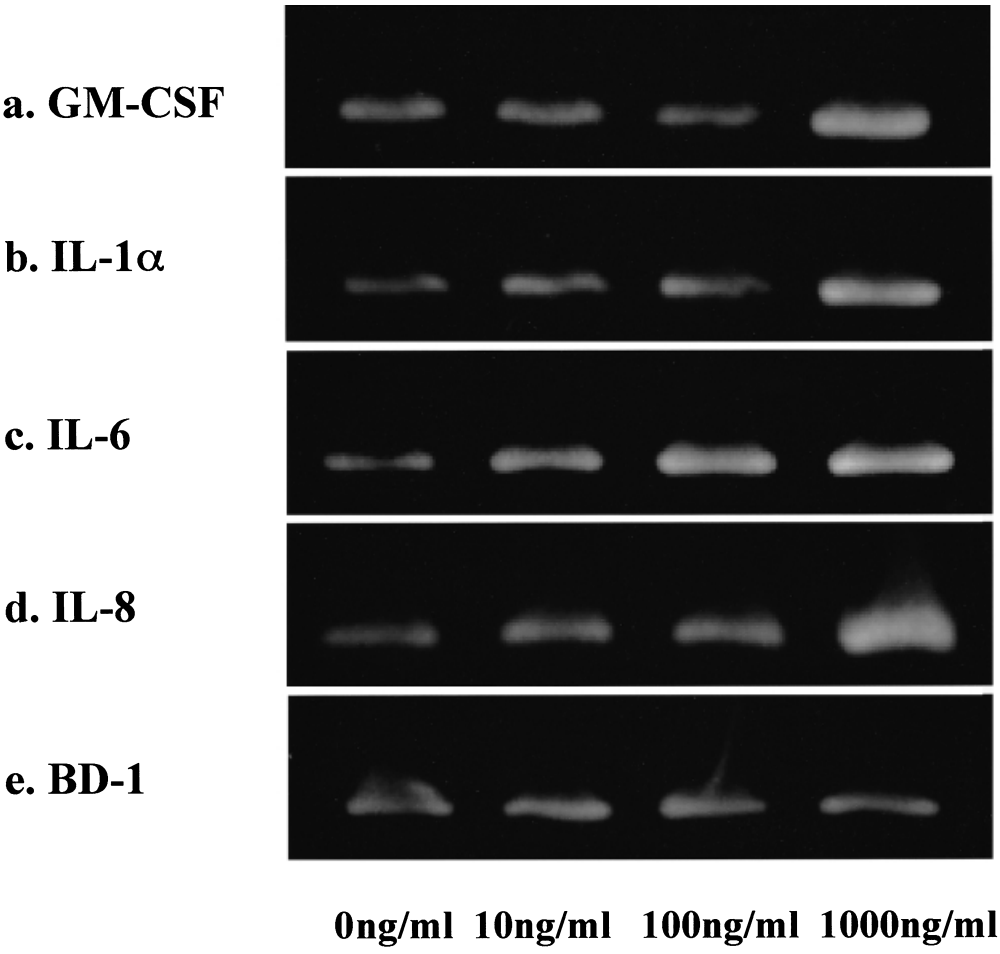

Fig. 3a–e. Expression of the mRNAs by epithelial cells on stimulation with lipopolysaccharide (*LPS*) at four concentrations in 6 h; 32 cycles. *From left to right*, 0 ng/ml (control), 10 ng/ml, 100 ng/ml, 1000 ng/ml. **a** GM-CSF, 32 cycles; **b** IL-1α, 32 cycles; **c** IL-6, 34 cycles; **d** IL-8, 33 cycles; **e** BD-1, 34 cycles

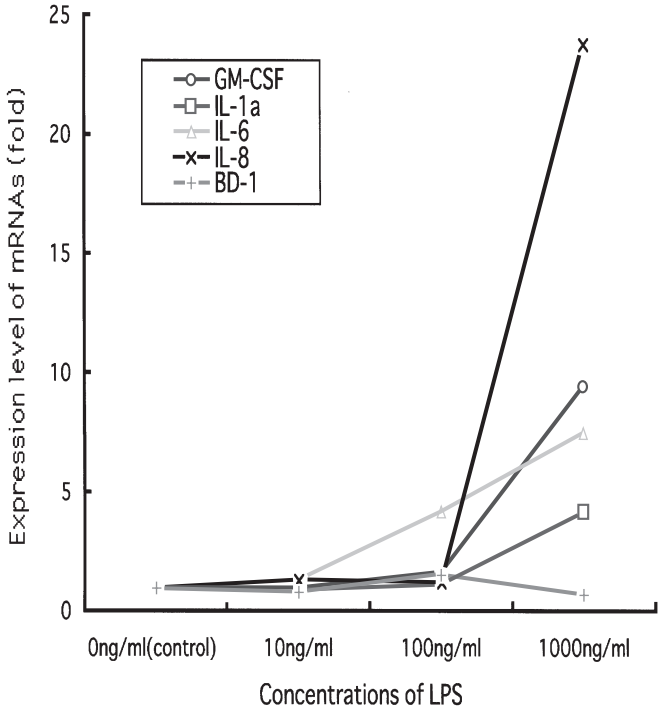

Fig. 4. Expression of mRNAs by epithelial cells on stimulation with different concentrations of LPS in 6 h. *From left to right*, 0 ng/ml (control), 10 ng/ml, 100 ng/ml, 1000 ng/ml (all data were standardized by G3PDH and divided by control value)

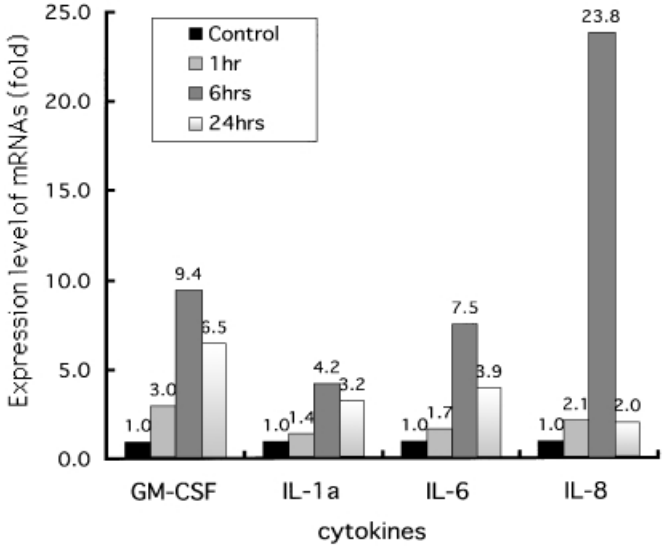

Fig. 5. Expression of mRNAs by epithelial cells with three different incubation times. Concentration of LPS, 1000 ng/ml. The expression was upregulated in a time-dependent manner (all data were standardized by G3PDH and divided by control value)

1α, IL-β, IL-6, IL-8, and TNF-α.[7] Our results are consistent with this previous study.[7]

IL-1 was suggested to induce keratinocyte proliferation and the induction of IL-1 itself and IL-6.[17] IL-1 and IL-6 are potent modulators of osteolysis.[18,19] Overexpression of these cytokines in epithelial rests of Malassez may induce their proliferation, and be involved in bone destruction when radicular cysts expand. The upregulation of these cytokines by LPS in the present study implies that overexpression of the cytokines is induced by bacterial infection. In fact, oral bacteria, such as *Porphyromonas gingivalis*, stimulated the upregulation of IL-1, IL-6, and IL-8 expression in oral epithelial cells.[20] IL-8 acts as a chemotactic factor of neutrophils, and is also induced by IL-1 production.[21] IL-6 and IL-8 may be upregulated directly and/or via IL-1 expression by LPS. Further studies need to be directed toward gaining an understanding of the mechanism of these cytokine expressions. GM-CSF is also secreted by other types of epithelial cells, and is involved in the regulation of dendritic cell function.[22] Langerhans cells, a type of dendritic cell, are often observed in the lining epithelium of radicular cysts.[23,24] GM-CSF released from the epithelial rests of Malassez may regulate Langerhans cell function in radicular cysts.

BD-1 is consistently produced in other types of epithelial cells under inflammatory conditions.[11,12] Our result, showing no alteration of BD-1 expression by stimulation with LPS, is consistent with previous reports.[11,12] Although BD-1 expression in the epithelial rests of Malassez may play no functional role under normal conditions, it is possible that the lining epithelium derived from the epithelial rests may take advantage of the BD-1 function to protect the epithelial rests from bacterial infection.

In summary, in the present study, we reported the expression of inflammatory cytokines and BD-1 in epithelial rests of Malassez in vitro. The results indicated that the epithelial rests of Malassez may actively participate in the inflammatory responses to bacterial infection, and that they play an important role in the defense mechanism of the radicular cyst.

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