Effects of periodontopathic bacteria on the expression of endothelin-1 in gingival epithelial cells in adult periodontitis

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ABSTRACT

Adult periodontitis, which is the major cause of adult tooth loss, is commonly characterized by chronic inflammatory disease caused by infection with periodontopathic bacteria such as *Porphyromonas gingivalis*. Our aims in the present study were to examine the expression of endothelin-1 (ET-1) in cultured HEp-2 epithelial cells after infection with *P. gingivalis*, and in gingival tissue from adult periodontitis patients. The cell lines were infected with the strains *P. gingivalis* 33277 and 381 for assessment of bacterial invasion using an antibiotic protection assay, and the expression of ET-1, inflammatory cytokines and cell adhesion molecules was examined by ELISA and reverse transcription–PCR. The expression of ET-1, as well as that of interleukin-1β, interleukin-8 and ICAM-1 (intercellular cell adhesion molecule 1), was induced significantly in a time-dependent manner, whereas the expression of MCP-1 (monocyte chemotactic protein-1), RANTES (regulated upon activation, normal T-cell expressed and secreted) and VCAM-1 (vascular cell adhesion molecule 1) was not. Furthermore, in gingival tissues from adult periodontitis patients, we also observed increased expression of ET-1 mRNA compared with tissue from normal healthy donors. These results suggest that infection by periodontopathic bacteria up-regulates the expression of ET-1, together with that of inflammatory cytokines and ICAM-1, in gingival epithelial cells, and that ET-1 expression may be closely involved in the regulation of cytokine responses and cell–cell adhesion in adult periodontitis lesions.

INTRODUCTION

Adult periodontitis (AP) is a chronic inflammatory disease induced by Gram-negative bacteria in subgingival pockets. *Porphyromonas gingivalis* is thought to be one of the major pathogens of periodontitis [1,2]. During the initiation and progression of periodontal disease, inflammatory cytokines are considered to play important roles. Several reports have suggested a relationship between the progression of periodontitis and the expression of interleukin-1 (IL-1), IL-6, IL-8 and tumour necrosis factor α in gingival tissues [3,4]. One of the cell adhesion molecules, intercellular adhesion molecule 1 (ICAM-1), has been detected in gingival tissues from AP patients, and it was suggested that ICAM-1 contributes to the retention and activation of inflammatory cells through cytokines and other chemical mediators [5,6]. On the other hand, in general, several inflammatory cytokines, such as IL-1, IL-6 and IL-8, are known to up-regulate the production of endothelin-1 (ET-1) [7]. ET-1

Key words: cell adhesion molecule, endothelin-1, inflammatory cytokine, *Porphyromonas gingivalis*.

Abbreviations: AP, adult periodontitis; ET-1, endothelin-1; ICAM-1, intercellular cell adhesion molecule 1; IL-1 (etc.), interleukin-1 (etc.); MCP-1, monocyte chemotactic protein-1; RANTES, regulated upon activation, normal T-cell expressed and secreted; RT-PCR, reverse transcription–PCR.

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was originally identified as a potent 21-residue vasoconstrictor peptide in vascular endothelial cells [8], and it is known to be produced by many different tissues, including the airway epithelium [9]. Most recently, we found that ET-1 was expressed in the periodontal epithelial cells of gingival tissue from AP patients [10]. Another study has shown that interactions of ET-1 and other cytokines may be involved in susceptibility to AP [11]. However, little information is known about the pathophysiological role of ET-1 in periodontal disease. In the present study, we tested ET-1 expression and the up-regulation of cytokine responses and cell adhesion molecules in an in vitro model of P. gingivalis infection using cultured epithelial cells.

METHODS

Bacterial strains
P. gingivalis A.T.C.C. 33277 and 381 were kindly provided by T. Koga (Kyushu University, Japan), and were grown anaerobically (5% CO₂, 85% N₂) in brain/heart infusion broth (BHI; Difco Laboratories, Detroit, MI, U.S.A.) supplemented with yeast extract (0.5%), hemin (5 μg/ml) and menadione (0.5 μg/ml).

Cell cultures
The human epithelial cell line HEp-2 was used in this study. HEp-2 cells were grown at 37 °C with 5% CO₂ in minimum essential medium supplemented with glutamine and 5% (v/v) fetal bovine serum.

Infection of epithelial cells
Approx. 10⁶ cells were washed three times with PBS and then infected by the addition of a resuspended overnight culture of 10⁷ P. gingivalis (A.T.C.C. 33277 or 381) in 1.0 ml of antibiotic-free medium at 37 °C. After 120 min of aerobic incubation, the monolayers were washed three times with PBS to remove extracellular bacteria. Medium containing gentamicin (300 μg/ml) and metronidazole (200 μg/ml) was then added to each well, and the plates were incubated for an additional 60 min aerobically at 37 °C. After incubation, the supernatants were collected for detection of ET-1, IL-8 and β-chemokines, including RANTES (regulated upon activation, normal T-cell expressed and secreted) and MCP-1 (monocyte chemotactic protein-1), by ELISA. The cells were then washed three times with PBS and used for RNA extraction.

Reverse transcription–PCR (RT-PCR) analysis
Cellular RNA was isolated using an RNeasy Mini Kit (QIAGEN) according to the manufacturer’s protocol. cDNA was synthesized from total RNA using the SUPERSCRIPT First-Strand Synthesis System for RT-PCR (GIBCO-BRL) according to the manufacturer’s instructions. One-tenth of the resulting cDNA mixture was then amplified in a solution containing 10× PCR buffer, 2.5 mM (each) dNTP, 40 pM (each) 5’ and 3’ primers, and 1 unit of Taq polymerase (Promega). Specific primers were designed from the known sequences of ET-1, ICAM-1 and the β-actin gene. The primers used in this study were as follows: ET-1: sense, 5’-TGCTCCCTGCTGTCCTTGATGATAAAGA-GTGTGTGTC-3’; antisense, 5’-GGTCACATAAGCCTCTCTGGAGGCTT-3’; ICAM-1: sense, 5’-TGACCATCTACAGCTTTCGGC-3’; antisense, 5’-AGCCCTGGCCAATTTGGAGCTG-3’; β-actin: sense, 5’-GGGGAGAAGATGACCCAG-3’; antisense, 5’-ATCACCATGCCAAGTGGTAC-3’. PCR for IL-1β and VCAM-1 (vascular cell adhesion molecule 1) was performed using a commercial RT-PCR kit (Ambion) according to the manufacturer’s instructions. The PCR reaction was initiated by denaturing the cDNA by heating at 94 °C for 1 min, annealing the primers at 55 °C for 2 min, and extending the primers at 72 °C for 1 min. The cycle was repeated 22–25 times by a programmable thermal controller. After the final cycle, the temperature was maintained at 72 °C for 10 min to allow reannealing of the amplified products. The amplified PCR products were then analysed by electrophoresis on 2% (w/v) agarose gels, and visualized by staining with ethidium bromide. The DNA bands were quantified using the computer software Quantity One (Bio-Rad).

ELISA for ET-1 and chemokines
The concentrations of ET-1 in the P. gingivalis-infected and mock-infected culture supernatant were determined using the Parameter ELISA system (R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer’s protocol. Absorbance was measured at 450 nm, using a microplate reader with a wavelength correction set at 650 nm. ELISA for IL-8 and chemokines was performed using a commercial human ELISA kit (ENDOGEN).

Tissue specimens
Gingival tissues with the presence of bleeding on probing and a probing depth greater than 6 mm were obtained from six patients with AP at periodontal surgery. The patients consisted of two men and four women, with a mean age of 59 years (range 39–71 years). Four gingival tissue specimens from a healthy site, ascertained using both clinical features and histological analysis, were obtained simultaneously. None of the subjects had any systemic diseases, and denied any intake of medication for at least 3 months prior to evaluation. The gingival
tissue was frozen immediately in liquid nitrogen and stored at −80 °C until RNA extraction.

Statistical analysis
Comparison between the expression of ET-1 and chemokines in control and bacterially infected cells was performed using the Mann–Whitney U test. P values of < 0.05 were considered significant.

RESULTS
Epithelial cells were co-cultured with *P. gingivalis* for 2 h. During the subsequent incubation, extracellular bacteria were removed and fresh medium containing antibiotics was added to kill the remaining extracellular bacteria. At various time points, total RNA was isolated from the epithelial cell cultures for RT-PCR analysis. As shown in Figure 1, *P. gingivalis* up-regulated the mRNA levels of IL-1β, ICAM-1 and ET-1. ET-1 mRNA, as well as IL-1β and ICAM-1 mRNAs, were present in uninfected cells, but the levels increased steadily up to 16 h post-infection. Next we examined ET-1 protein expression by ELISA (Figure 2). ET-1 protein was not detected in uninfected cells, and levels increased slowly after infection. The levels peaked at 16 h and then declined thereafter. Compared with controls, infection with *P. gingivalis* 33277 induced a 2.3-fold increase in the production of ET-1 at 16 h post-infection. On the other hand, IL-8 protein levels had increased by 3-fold at 16 h post-infection, but RANTES, one of the β-chemokines, was not induced significantly. Another β-chemokine measured here, MCP-1, also remained suppressed (results not shown).

Essentially similar results were obtained in experiments using *P. gingivalis* 381 (results not shown).

Figure 3 shows the mRNA expression of ET-1 in small gingival tissue samples obtained from patients with AP and normal healthy subjects, as assessed using RT-PCR. ET-1 mRNA was expressed not only in all inflamed gingival tissues, but also weakly in normal gingival tissues. The level of ET-1 expression in gingival tissues from AP patients was increased compared with that in tissues from normal healthy subjects.
DISCUSSION

In the present study, we have shown that expression of ET-1 was up-regulated following P. gingivalis infection in cultured epithelial cells. This is the first report suggesting that ET-1 is involved in the up-regulation of inflammatory responses to P. gingivalis. ET-1 is known to have multifunctional regulatory activities, and is found in various tissues [8]. We showed recently that ET-1 is expressed in the gingival epithelium [10]. Our aims in the present study were to investigate whether ET-1 is induced in cultured epithelial cells after infection with P. gingivalis, and if ET-1 could be associated with severity of AP.

To date, there have been several reports on the up-regulation of ET-1 after infection. In vitro infection experiments using respiratory syncytial virus, the expression of ET-1 was up-regulated along with that of 5-lipoxygenase, the key pro-inflammatory enzyme of the leukotriene pathway. This may mediate the consequent exacerbation of the inflammatory process [12]. In experiments using rats, the intragastric application of Helicobacter pylori lipopolysaccharide elicited a 3.1-fold enhancement in the mucosal expression of ET-1, combined with a loss of compensatory action by IL-4 [13]. From the results of our present study, it is apparent that the up-regulation of ET-1 expression is associated with the interplay between other mediators, such as the cytokines and cell adhesion molecules.

The periodontal pathogen P. gingivalis has been observed within gingival tissues in vivo. P. gingivalis has been shown to bind to and invade KB epithelial cells [14], as well as primary cultures of pocket epithelium [15]. Several reports have suggested a relationship between the progression of periodontitis and the expression of IL-1, IL-6, IL-8 and tumour necrosis factor z in gingival crevicular fluid or in gingival tissues [3,4]. On the other hand, P. gingivalis up-regulated IL-8 and ICAM-1 mRNAs in cultured human gingival epithelial cells [6]. Our present study suggests a positive relationship between the expression of ET-1 and ICAM-1. It will be of interest to investigate the mechanisms that govern the regulation of these two molecules in bacterially infected gingival epithelial cells.

As described above, the results presented here suggest that ET-1 is deeply involved in the inflammatory events of periodontitis. This work may have clinically important implications. A recent study showed that ET levels in saliva are raised in patients with chronic heart failure and indicate progression of disease severity [16]. These findings suggest that measurement of ET in saliva may be a simple, non-invasive method to assist in the diagnosis and assessment of disease severity in patients with suspected or established chronic heart failure [16]. From such a standpoint, ET-1 in gingival tissues could be used as a marker for the diagnosis and assessment of disease severity in periodontitis.

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REFERENCES