Abstract: The junctional epithelium and dental enamel adhere because of hemidesmosomes containing laminin 5 and α6β4 integrin, which are important adhesion molecules in the internal basal lamina. Interleukin (IL)-1 is important in the pathogenesis of periodontal disease. IL-1β induces bone resorption by activating osteoclasts; however, its effects on adhesion of epithelial cells remain to be clarified. Laminin β3, β4 integrin, and focal adhesion kinase mRNA levels were higher after 1 h and 3 h of stimulation with IL-1β (1 ng/mL), and IL-1β, type I α1, and type IV α1 collagen mRNA levels were higher after 1 h and lower after 3 h of stimulation with IL-1β. After IL-1β stimulation, colocalization of laminin 5 and β4 integrin was increased after 1 h, colocalization of β4 integrin and plectin was increased after 1 h and decreased after 3 h, and colocalization of β4 integrin and type IV collagen was decreased after 3 h. Wound healing assays showed that IL-1β treatment (3 h) delayed wound healing. These results suggest that IL-1β enhances cell adhesion by altering localization of epithelial adhesion molecules.

Keywords: basal lamina; cell adhesion; hemidesmosome; inflammatory cytokine; junctional epithelium.

Introduction

The gingival epithelium comprises the oral epithelium, sulcular epithelium, and junctional epithelium (JE). The JE forms apical to the dento-epithelial junction to the sulcus. The coronal end of the JE forms the bottom of the gingival sulcus and overlaps with the sulcular epithelium (1,2). The JE has two basal laminas—the internal basal lamina faces the tooth and the external basal lamina faces the gingival connective tissue. Hemidesmosomes are involved in promoting adhesion of epithelial cells to the underlying basement membrane in stratified and other complex epithelia (4,5). The basal lamina is composed of extracellular matrix containing laminin, proteoglycan, and type IV collagen. However, laminin 5 is found only in the internal basal lamina, which lacks type IV collagen, and both these proteins are present in the extracellular matrix. Laminin 5 consists of α3, β3, and γ2 subunits and contributes to cell adhesion associated with α6β4 integrin at hemidesmosomes. Laminin 5 and α6β4 integrin were reported to be involved in wound healing of epithelial tissues (6-9). Plectin, a functionally and universally versatile cytolinker protein, has been implicated in hemidesmosome functions, as early binding of plectin to the β4 subunit of α6β4 integrin is a critical step for hemidesmosome formation, and regions of plectin were shown to be involved in the interaction (10,11).

Interleukin (IL)-1β is an essential mediator of proinflammatory response (12) and is involved in defense against external challenges, such as microorganisms, environmental irritants, and injury, and in innate immunity and periodontitis pathophysiology (13). In adherent

Original

IL-1β enhances cell adhesion through laminin 5 and β4 integrin in gingival epithelial cells

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fibroblasts, IL-1β induces release of matrix metalloproteinases (MMPs), which remodel the extracellular matrix (14,15) and enhance tissue repair after short-term perturbations of connective tissue homeostasis, such as acute injury (16-18). IL-1β has a central role in tissue destruction in many chronic inflammatory diseases, such as rheumatoid arthritis, pulmonary and cardiac fibrosis, inflammatory bowel disease, type 2 diabetes, and periodontitis (19,20). In this study, we analyzed the effects of IL-1β on adhesion molecules in gingival epithelial cells.

**Materials and Methods**

**Reagent**

Rabbit polyclonal antibodies (laminin 5 and type IV collagen), mouse monoclonal antibodies (β4 integrin), Phalloidin-iFluor 488 reagent, goat anti-rabbit Alexa 488, and goat anti-mouse Alexa 647 were obtained from Abcam (Tokyo, Japan). Rabbit polyclonal antibodies to plectin were purchased from Novus (Littleton, CO, USA). Human recombinant IL-1β and alpha-minimum essential medium (αMEM) were purchased from Wako (Tokyo, Japan). Fetal calf serum (FCS), penicillin and streptomycin, TrypLE Express, and TRIzol Reagent were purchased from Invitrogen (Carlsbad, CA, USA). The PrimeScript RT reagent kit and SYBR Premix Ex Taq II were purchased from Takara Bio (Tokyo, Japan). Bovine serum albumin (BSA), complete protease inhibitor cocktail, and phenylmethylsulfonyl fluoride were purchased from Sigma Aldrich Japan (Tokyo, Japan). Anti-mouse IgG (whole molecule) peroxidase antibody produced in rabbit, anti-rabbit IgG (whole molecule) peroxidase antibody produced in goat, and ECL Prime Western Blotting Detection Reagents were purchased from GE Healthcare (Buckinghamshire, UK). All chemicals used were of analytical grade.

**Cell cultures**

Human gingival epithelial Ca9-22 cells were cultured in αMEM containing 10% FCS until 70-80% confluent in 5% CO2 and 95% air at 37°C. Ca9-22 cells were stimulated with IL-1β (1 ng/mL) for 1 h and 3 h.

**Real-time PCR**

Total RNA was isolated by TRIzol Reagent according to the manufacturer’s protocol. Total RNA (1 μg) was used as a template for cDNA, which was prepared with the PrimeScript RT reagent kit. Using SYBR Premix Ex Taq II in a TP800 Thermal Cycler Dice Real-Time System (Takara Bio), we performed quantitative real-time PCR with the following primer sets: β4 integrin forward, 5'-CTCCACCGAGTCAAGCTTC-3'; β4 integrin reverse, 5'-CGGGTAGTTGCTTGTCCGTGTA-3'; laminin α3 chain forward, 5'-TGCTAACAGTATCAGGAGATCT-3'; laminin α3 chain reverse, 5'-CTTGAGTTCAAGCCATTGGCC-3'; laminin β3 chain forward, 5'-CCAAGGCTGAGACCTACTGC-3'; laminin β3 chain reverse, 5'-GAATCTCCTGCTCAGGTCCA-3'; laminin γ2 chain forward, 5'-GACAAACTGGTAATGGATTGAGC-3'; type I α1 collagen forward, 5'-ACCTGCTTCAAATCTCCTTG-3'; type I α1 collagen reverse, 5'-AGGGTGTTGTGAAGCCATTGAG-3'; MMP3 forward, 5'-CTGGCCAGGGATTAATGGAG-3'; MMP3 reverse, 5'-CAATTTCATGAGCAACGAGA-3'; tissue inhibitor of metalloproteinase-1 (TIMP1) forward, 5'-GCGGTTCATCTCAGGGACAGCAG-3'; TIMP1 reverse, 5'-GTCGTCACAGGGACATGAG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-GCACCAGTCAGTGAAGAC-3'; GAPDH reverse, 5'-ATGGGTGTTGACAGCCAGTGA-3'. The amplification reactions were performed in 25 μL of final volume containing ×2 SYBR Premix EX Taq (12.5 μL), 0.4 μm forward and reverse primers (0.2 μL), and 70 ng cDNA (7 μL) for β4 integrin, laminin α3, laminin β3, laminin γ2, type IV α1 collagen, type I α1 collagen, FAK, MMP3, and TIMP1, and 50 ng cDNA (5 μL) for GAPDH. To reduce variability between replicates, PCR premixes containing all reagents except for cDNA were prepared and aliquoted into 0.2-mL PCR tubes (Nippon Genetics). The thermal cycling conditions were 10 s at 95°C, 45 cycles of 5 s at 95°C, and 30 s at 60°C. Post-PCR melting curves confirmed the specificity of single-target amplification, and expressions of β4 integrin, laminin α3, laminin β3, laminin γ2, type IV α1 collagen, type I α1 collagen, FAK, MMP3, and TIMP1 relative to GAPDH were determined in triplicate.

**Wound healing assay**

Ca9-22 cells were cultured in αMEM containing 10% FCS on fibronectin (10 μg/mL)-coated 35-mm culture dishes and grown to confluence. After the medium was changed to αMEM containing 1% FCS, cell layers were scratched at a diameter of about 3 mm with a 1-mm-wide cell scraper (Corning) in the center of the dishes and washed twice with phosphate-buffered saline (PBS) to remove the detached cells. The cells were stimulated
with IL-1β (1 ng/mL) for 1 h and 3 h and then cultured for 6 days to analyze the wound healing area.

**Western blot**
Ca9-22 cells were lysed, and equal amounts of protein were loaded and separated on 8% SDS-PAGE gels and transferred to Hybond 0.2-μm polyvinylidene fluoride membranes. The membranes were blocked and probed with the various primary antibodies at 4°C overnight, followed by fluorescent secondary antibody incubation for 1 h at room temperature. The membrane was incubated with anti-β4 integrin (ab29042; Abcam, Cambridge, UK), anti-laminin 5 (ab14509; Abcam), type IV collagen (ab6586; Abcam), and anti-α tubulin (sc5286; Santa Cruz Biotechnology, CA, USA) antibodies for 2 h. Anti-rabbit and anti-mouse IgG conjugated with horse-radish peroxidase were used as the secondary antibodies. Immunoreactivities were detected by ECL Prime Western Blotting Detection Reagents. An ImageQuant LAS 4000 system (GE Healthcare, Little Chalfont, Buckinghamshire, England) was used for detection and analysis of immunoblots.

**Immunofluorescent colocalization analysis**
An 8-well plate was seeded with 1 × 10^4 Ca9-22 cells per well, and the cells were cultured in αMEM with 10% FCS for 12 h. Countess Cell Counting Chamber Slides (Invitrogen) were used for cell counting. Cells were washed with PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 1.5 mM KH₂PO₄; pH 7.4). Eight-chamber slides (Corning) were coated with 10 μg/mL of fibronectin at 37°C for 60 min. The medium was changed to αMEM with 1% FCS for 3 h, and the cells were then treated with 1 ng/mL IL-1β each for 1 h and 3 h. After stimulation, cells were fixed in 4% paraformaldehyde for 10 min. After three washes with PBS, the cells were treated with 0.1% Triton X-100 for 5 min for permeabilization. Cells were blocked in 2.5% goat serum in 4% BSA for 20 min at room temperature. After one wash with PBS, the primary antibodies rabbit polyclonal anti-laminin 5, anti-type IV collagen, and anti-plectin antibody, and mouse monoclonal anti-integrin β4 were used at a 1:200 concentration for 2 h at 37°C. After three washes with PBS, the secondary antibodies Alexa Fluor 488 and Alexa Fluor 647 goat anti-rabbit IgG were used at a 1:200 concentration for 1 h at room temperature. After three washes with PBS, coverslips were mounted with AntiFade Poly/Mount with DAPI (Polysciences, Warrington, PA, USA). Fluorescent images were viewed under a Zeiss LSM 510 (Oberkochen, Germany) confocal microscope.

**Statistical analysis**
In all experiments, separate assays were repeated at least three times. For quantitative data, means ± SEM was computed. Comparisons of multiple samples were analyzed with ANOVA. Statistical significance was set at P < 0.05.

**Results**

**Regulation of adhesion molecule mRNA levels by IL-1β**
To determine whether IL-β affects mRNA levels of cell adhesion molecules in Ca9-22 cells, we stimulated Ca9-22 cells with IL-1β (1 ng/mL) for 1 h and 3 h. β4 integrin, laminin β3, and FAK mRNA levels increased after 1 h and 3 h of IL-1β stimulation (Fig. 1A-C). Laminin α3 and laminin γ2 mRNA levels increased after 1 h of IL-1β stimulation (Fig. 1D, E). MMP3 and TIMP1 mRNA levels increased after 3 h of IL-1β stimulation (Fig. 1F, G). Type IV α1 and type I α1 collagen mRNA levels increased after 1 h and decreased after 3 h of IL-1β stimulation (Fig. 1H, I).

**Effect of IL-1β on cell adhesion and protein production**
To determine colocalization of cell adhesion molecules, Ca9-22 cells were immunostained with antibodies of cell adhesion molecules, and the extent of colocalization was quantified by Pearson correlation analysis (21). Colocalization of laminin 5 and β4 integrin in Ca9-22 cells increased after 1 h of IL-1β stimulation (Fig. 2A, B). Colocalization of β4 integrin and plectin increased after 1 h and decreased after 3 h of stimulation (Fig. 2C, D). Colocalization of β4 integrin and type IV collagen decreased after 3 h of IL-1β stimulation (Fig. 2E, F). Western blot showed that type IV collagen protein levels (300 kDa) increased after 3 h of IL-1β stimulation, whereas β4 integrin (202 kDa) and laminin 5 (100-150 kDa) protein levels were unchanged after IL-1β stimulation (Fig. 3A-C).

**Effect of IL-1β on functional analyses of wound healing**
Mean wound healing area was significantly smaller in culture dishes treated with IL-1β for 1 h, and significantly larger in dishes treated with IL-1β for 3 h, than in control dishes (Fig. 4).

**Discussion**
JE is an epithelial component that is directly attached to the tooth surface and helps protect against periodontopathic bacteria. Periodontitis is a chronic inflammatory
A disease caused by bacterial, environmental, and host factors. It results in progressive destruction of tooth supporting structures (22). Colocalization analyses showed that adhesion was stronger in the internal basal lamina than in the external basal lamina, as indicated by the present findings that colocalization of laminin 5 and β4 integrin increased after 1 h of IL-1β stimulation and colocalization of β4 integrin and type IV collagen decreased after 3 h of IL-1β stimulation (Fig. 2A, B, E, F). These results support the findings of previous studies (2,3) and suggest that IL-1β induced by oral bacteria might increase pathogen resistance in the internal basal lamina. Immunofluorescence showed protein expressions of laminin 5 and type IV collagen in the extracellular

Fig. 1 Effects of IL-1β on mRNA levels of adhesion molecules in Ca9-22 cells. mRNA levels of β4 integrin (A), laminin β3 (B), FAK (C), laminin α3 (D), laminin γ2 (E), MMP3 (F), TIMP1 (G), Type IV α1 collagen (H), and Type I α1 collagen (I) in Ca9-22 cells were quantified by real-time PCR. Cells were treated with IL-1β (1 ng/mL) for the indicated times. GAPDH was used as the loading control. The data represent the results of three separate experiments. ***P < 0.001, **P < 0.01, *P < 0.05
matrix (Fig. 2A, E). Thus, it is important to investigate the components of extracellular matrix. Furthermore, colocalization of the intercellular adhesion molecules β4 integrin and plectin increased after 1 h of IL-1β stimulation (Fig. 2C, D). Evidence suggests that formation of β4 integrin-plectin and β4 integrin-laminin 5 complexes may be a biological defense mechanism. Pöllänen et al. showed that cytokeratin 17 is involved in the junctional epithelium—β4 integrin, plectin, type IV collagen, and laminin 5 levels were increased in periodontitis inflammatory sites (3). IL-1β increased the number of activated β1 integrin-stained focal adhesions in gingival fibroblasts and induced talin and paxillin in focal adhesions after IL-1β treatment (23). In the present study, β4 integrin,
laminin β3, and FAK mRNA levels increased after stimulation with IL-1β for 1 h and 3 h (Fig. 1A-C). In addition, laminin α3, laminin γ3, type IV α1, and type I α1 collagen mRNA levels increased after 1 h of IL-1β stimulation (Fig. 1D, E, H, I). Interestingly, changes in mRNA levels of type IV α1 and type I α1 collagen were similar after stimulation with IL-1β (Fig. 1H, I). β4 integrin, laminin β3, and FAK mRNA levels, which are involved in extracellular matrix formation, increased in response to IL-1β (Fig. 1A-C). Additionally, inflammatory cytokines such as TNF-α induced phosphorylation of FAK in human periodontal ligament fibroblasts (24). Schwanhäusser et al. (25) reported that the half-lives of mRNA and protein levels were not correlated and that cellular protein abundance was predominantly controlled at the level of translation. Similarly, the present results showed no correlation between mRNA and protein levels of β4 integrin, laminin 5, or type IV collagen (Fig. 1, 3). In wound healing assays, the mean wound healing area was significantly larger in dishes treated with IL-1β for 3 h than in the controls (Fig. 4). Cell migration is adversely affected by inflammatory cytokines for epithelial cells and gingival fibroblasts (26). In summary, the present results indicate that IL-1β alters colocalizations of laminin 5, plectin, and type IV collagen with β4 integrin.

Fig. 3 Western blot analyses for protein levels of β4 integrin, laminin 5, and type IV collagen. Ca9-22 cells were treated with 1 ng/mL IL-1β for 1 h and 3 h before lysis. Equal amounts of the proteins were loaded in each lane and probed with anti-β4 integrin, laminin 5, and type IV collagen antibodies. α-Tubulin was used as the loading control.

Fig. 4 Wound healing analyses on re-epithelialization. Re-epithelialization in Ca9-22 cells was analyzed on fibronectin (10 μg/mL)-coated dishes. Observation with an inverted microscope showed IL-1β-treated wounded cells after scratching. The panels show cells immediately after scraping (A) and 4 days later (B). Mean wound healing area (re-epithelialization) in IL-1β-treated dishes is shown in panel (C).

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**Conflict of interest**

The authors have no conflict of interest to declare.

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