T. Yoshimoto¹, T. Fujita^{1*}, K. Ouhara¹,
M. Kajiya¹, H. Imai¹, H. Shiba¹,
and H. Kurihara¹

¹Department of Periodontal Medicine, Division of Applied Life Sciences, Institute of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan; *corresponding author, tfuji@hiroshima-u.ac.jp

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ABSTRACT

Apoptosis is thought to contribute to the progression of periodontitis. It has been suggested that the apoptosis of epithelial cells may contribute to the loss of epithelial barrier function. Smad2, a downstream signaling molecule of TGF- β receptors (TGF- β Rs), is critically involved in apoptosis in several cell types. However, the relationship between smad2 and bacteria-induced apoptosis has not yet been elucidated. It is possible that the regulation of apoptosis induced by periodontopathic bacteria may lead to novel preventive therapies for periodontitis. Therefore, in the present study, we investigated the involvement of smad2 phosphorylation in apoptosis of human gingival epithelial cells induced by *Aggregatibacter actinomycetemcomitans* (*Aa*). *Aa* apparently induced the phosphorylation of smad2 in primary human gingival epithelial cells (HGEs) or the human gingival epithelial cell line, OBA9 cells. In addition, *Aa* induced phosphorylation of the serine residue of the TGF- β type I receptor (TGF- β RI) in OBA9 cells. SB431542 (a TGF- β RI inhibitor) and siRNA transfection for TGF- β RI, which reduced both TGF- β RI mRNA and protein levels, markedly attenuated the *Aa*-induced phosphorylation of smad2. Furthermore, the disruption of TGF- β RI signaling cascade by SB431542 and siRNA transfection for TGF- β RI abrogated the activation of cleaved caspase-3 expression and repressed apoptosis in OBA9 cells treated with *Aa*. Thus, *Aa* induced apoptosis in gingival epithelial cells by activating the TGF- β RI-smad2-caspase-3 signaling pathway. The results of the present study may suggest that the periodontopathic bacteria, *Aa*, activates the TGF- β RI/smad2 signaling pathway in human gingival epithelial cells and induces apoptosis in epithelial cells, which may lead to new therapeutic strategies that modulate the initiation of periodontitis.

KEY WORDS: epithelial cells, TGF- β type I receptor, cleaved caspase-3, periodontitis, gingival epithelium, periodontopathic bacteria.

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Smad2 is Involved in *Aggregatibacter actinomycetemcomitans*-induced Apoptosis

INTRODUCTION

Periodontitis is the most prevalent infectious disease caused by periodontopathic bacteria and is a chronic inflammatory disease. The gingival junctional epithelium, which is located at a strategically important interface between the gingival sulcus and host tissue, recognizes bacterial infections. Microbial adhesion to and invasion of the gingival epithelium are the initial events of periodontitis (Schroeder and Listgarten, 1997; Abuhussein *et al.*, 2013). Apoptosis in epithelial cells triggers the destruction of epithelial barrier function, which plays a crucial role in the onset and progression of periodontitis (Schroeder and Listgarten, 1997; Kato *et al.*, 2000; Dickinson *et al.*, 2011; Abuhussein *et al.*, 2013). Previous studies have reported that many TUNEL-positive cells were detected in the gingival epithelium of patients with periodontitis (Tonetti *et al.*, 1998; Abuhussein *et al.*, 2013). Therefore, the signaling cascade involved in micro-organism-induced apoptosis in gingival epithelial cells needs to be investigated in more detail and may lead to the development of novel preventive therapies for periodontitis.

TGF- β transmits signals by inducing TGF- β type II receptor (TGF- β RII) and TGF- β type I receptor (TGF- β RI) to form a tetrameric receptor complex, which leads to the activation of TGF- β RI. TGF- β RI, in turn, phosphorylates smad2, which translocates to the nucleus to regulate transcription (Derynck and Zhang, 2003). Smad2 is a well-known downstream signaling molecule of TGF- β Rs and plays a key role in TGF- β -mediated apoptosis in various cell types (Lee *et al.*, 2002; Wildey *et al.*, 2003; Mithani *et al.*, 2004; Ramjaun *et al.*, 2007; Yang *et al.*, 2009). An increase in the number of apoptotic-positive cells was recently observed in the gingival junctional epithelium in smad2 transgenic mice (Fujita *et al.*, 2012). Therefore, the marked acceleration of TGF- β RI/smad2 signaling by microbial pathogens may cause apoptosis and disrupt the epithelial barrier.

Aggregatibacter actinomycetemcomitans (*Aa*), a gram-negative facultative capnophilic anaerobe, can ferment many sugars, including glucose, fructose, and maltose. Previous studies identified numerous virulence factors from *Aa* that affected the gingival epithelium and triggered the onset of periodontitis, including lipopolysaccharide (LPS), leukotoxin, cytolethal distending toxin (CDT), collagenase, and outer membrane proteins (Wilson and Henderson, 1995; Schreiner *et al.*, 2003; Rogers *et al.*, 2007; Kachlany, 2010; Höglund Åberg *et al.*, 2013). *Aa* is recognized as the etiological pathogen for aggressive periodontitis and severe adult periodontitis, while being involved in other medical diseases such as thyroid and brain abscess, urinary tract infections, and sub-acute bacterial endocarditis. Furthermore, evidence to suggest that *Aa* induces apoptosis in gingival epithelial cells is increasing (Li *et al.*, 2011; Kang *et al.*, 2012).

Based on these findings, we hypothesized that *Aa* may activate TGF- β Rs/smad2 signaling to induce apoptosis in gingival junctional epithelial cells. We herein examined the involvement of smad2 signaling in cultured gingival epithelial cells stimulated by *Aa*.

MATERIALS & METHODS

Bacterial Strains and Culture

Aa strain Y4 (ATCC, Manassas, VA, USA) was grown in Todd-Hewitt broth supplemented with 1% yeast extract (TSBY; Difco Laboratories, Detroit, MI, USA) in humidified 5% CO₂ atmosphere at 37°C for 2 days. After cultivation, whole cells were fixed with 1% formalin at 4°C for 12 hr, harvested by centrifugation, and washed 3 times in phosphate-buffered saline (PBS, pH 7.4). Some of the washed *Aa* Y4 was suspended in Humedia-KB2 medium (pH 7.4, Kurabo, Osaka, Japan) containing 10 μ g/mL insulin, 5 μ g/mL transferrin, 10 μ M 2-mercaptoethanol, 10 μ M 2-aminoethanol, and 10 nM sodium selenite.

Cells and Cell Culture

Healthy gingival tissues, which had been surgically dissected through the process of third molar extraction and were going to be discarded, were collected with patients' informed consent. Human gingival epithelial cells (HGECS) were isolated as previously described, with minor modification (Uchida *et al.*, 2005; Fujita *et al.*, 2006, 2010). Briefly, gingival tissues were treated with dispase overnight at 4°C and then divided into the epithelium and connective tissue. The epithelium was treated with 0.025% trypsin and 0.01% EDTA for 15 min, minced, and treated with a trypsin inhibitor. The HGEC suspension was centrifuged at 120 $\times$ g for 5 min, and the pellet was suspended in Humedia-KB2 medium containing 10 μ g/mL insulin, 5 μ g/mL transferrin, 10 μ M 2-mercaptoethanol, 10 μ M 2-aminoethanol, 10 μ M sodium selenite, 50 μ g/mL bovine pituitary extract, 100 units/mL penicillin, and 100 μ g/mL streptomycin. The cells were seeded in 60-mm plastic tissue culture plates coated with type I collagen, and incubated in 5% CO₂/95% air at 37°C. When the cells reached subconfluence, they were harvested and subcultured.

OBA9 cells, a Simian virus-40 (SV40) antigen-immortalized human gingival epithelial cell line, were kindly given by Professor Shinya Murakami (Osaka University) (Kusumoto *et al.*, 2004). OBA9 cells were cultured with Humedia-KB2 medium containing 10 mg/mL insulin, 0.1 mg/mL hEGF, 0.67 mg/mL hydrocortisone hemisuccinate, 50 mg/mL gentamycin, 50 mg/mL amphotericin B, and 2 mL BPE (medium A). Before the addition of *Aa* Y4, these cells were incubated in medium without growth factor (medium B) for 3 hr. The cells were then pre-treated for 30 min with or without SB431542 (TGF- β RI inhibitor, 10 μ M, R&D Systems, Minneapolis, MN, USA) and then treated with *Aa* Y4 for various periods. Regarding chemical reagents dissolved in dimethylsulfoxide, an appropriate concentration of dimethylsulfoxide was added as a solvent control.

Western Blotting

Proteins were separated by SDS-PAGE and transferred to a nitrocellulose (NC) membrane (Bio-Rad Laboratories, Hercules, CA, USA). After being blocked with 5% nonfat milk in TBST for 1 hr, the membrane was washed and incubated with the primary antibody, rabbit anti-human cleaved caspase-3 antibody, rabbit anti-human phosphorylated smad2 (Ser465/467) antibody, rabbit anti-human TGF- β RI antibody, mouse anti-human β -actin antibody (Cell Signaling Technology, Beverly, MA, USA), and mouse anti-human total smad2/3 antibody (BD Transduction Laboratories, San Jose, CA, USA). The membrane was washed 3 times and incubated with a peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG antibody (R&D Systems) for 1 hr at room temperature. Immunodetection was performed according to the manual supplied with the ECL Prime Western blotting detection reagents (BioRad Laboratories, Hercules, CA, USA).

Immunoprecipitation

Recombinant human TGF- β 1 was obtained from R&D Systems. The TGF- β RI protein in the cell lysate was immunoprecipitated with rabbit anti-TGF- β RI polyclonal antibody (Abcam, Cambridge, MA, USA) that was pre-bound to the Crosslink Magnetic IP and Co-IP Kit (Thermo Scientific, Rockford, IL, USA). After being extensively washed with the lysis buffer, proteins captured by anti-TGF- β RI antibody-coated beads were separated by SDS-PAGE and subjected to Western blotting with rabbit anti-phosphoserine antibody (Abcam) or with rabbit anti-TGF- β RI polyclonal antibody (Cell Signaling Technology). After the membrane was reacted with HRP-conjugated donkey anti-rabbit IgG (R&D Systems), immunodetection was performed as described above.

Real-time PCR Assay

First, standard cDNA synthesis was performed with 1 μ g of total RNA extract in a total volume of 20 μ L (Roche, Tokyo, Japan). Real-time PCR was performed with a Lightcycler system using SYBR green (Roche). The sense and anti-sense primers for human TGF- β type I receptor were as follows: (sense) 5'-TGG TCTTGCCCATCTTCACA-3' and (antisense) 5'-ATTGCATAGATGTCAGCACG-3'; and GAPDH mRNA (sense), 5'-AACG TGTCAGTGGTGGACCTG-3' and (anti-sense) 5'-AGTGGGT GTCGCTGTTGAAGT-3'.

Apoptosis Assay

TUNEL staining for apoptotic cells was performed with a Dead-End fluorometric TUNEL system (Promega, Madison, WI, USA). Fluorescence signals were detected with an Olympus FSX100 fluorescence microscope (Olympus, Tokyo, Japan).

Small Interfering RNA (siRNA) Knockdown of the TGF- β Type I Receptor

Validated TGF- β RI siRNA and negative-control siRNA were obtained from Invitrogen (Carlsbad, CA, USA) (identification

Figure 1. *Aa* Y4 facilitated the phosphorylation of smad2 in human gingival epithelial cells (HGECS). **(A)** HGECS were exposed to *Aa* Y4 at 10⁶, 10⁷, 10⁸, and 10⁹ cells/well (MOI: 1, 10, 10², and 10³) for 1 hr. **(B)** HGECS were exposed to *Aa* Y4 at 10⁷ cells/well for various periods. **(C)** OBA9 cells were exposed to *Aa* Y4 at 10⁷ cells/well for the indicated periods. Phosphorylated (p) and total (t) smad2 levels were analyzed through Western blotting. Band densities were quantified through densitometric scanning of each band with the National Institutes of Health Image J software. The graph indicates the ratio of phosphor-smad2/total smad2. Values represent the mean $\pm$ SD of 3 cultures. * $p < .05$, ** $p < .01$ values differ significantly (t test).

nos. HSS110697 for TGF- β RI siRNA, HSS106251 for smad2 siRNA, and 12935-300 for negative-control siRNA). OBA9 cells were seeded at a density of 2.0×10^4 cells *per* well in 6-well plastic culture plates and cultured in medium A for 24 hr at 37°C. In total, 30 nM of TGF- β RI siRNA and negative control siRNA were transfected into the cells with lipofectamine RNAiMAX reagent (Invitrogen), according to the manufacturer's instructions. Cells treated with or without stimulants were collected after 48 hrs of incubation.

Statistical Analysis

Comparisons between and among groups were analyzed with the Student's t test.

RESULTS

To determine whether *Aa* Y4 was involved in the phosphorylation of smad2 in gingival epithelial cells, we exposed HGECS to *Aa* Y4 at 10⁶, 10⁷, 10⁸, and 10⁹ cells *per* well (MOI: 1, 10, 10², 10³) for 1 hr. *Aa* Y4 (MOI: 1, 10, 10², 10³) significantly induced the phosphorylation of smad2 in HGECS (Fig. 1A). Based on this result, we used *Aa* Y4 at 10⁷ cells *per* well (MOI: 10) in the following experiments. HGECS exposed to *Aa* Y4 induced the phosphorylation of smad2, peaking at 1 hr and then gradually decreasing (Fig. 1B). We used OBA9 cells in subsequent experiments to examine the relationship between *Aa* Y4 and the phosphorylation of smad2 in gingival epithelial cells in more detail. OBA9 cells were exposed to *Aa* Y4 for various periods. Similar to the results obtained with HGECS, *Aa* Y4 also markedly facilitated the phosphorylation of smad2 in OBA9 cells for 1 hr (Fig. 1C). We then assessed the activation of TGF- β RI in OBA9 cells based on these results, to determine whether the *Aa* Y4-enhanced phosphorylation of smad2 was mediated through TGF- β RI. In general, in response to the binding of TGF- β 1 to TGF- β Rs, the interaction between TGF- β RII serine kinase and TGF- β RI elicits the phosphorylation of serines (187, 189, 191) in the GS domain of TGF- β RI, which, in turn, induces smad2 signaling (Derynck and Zhang, 2003). The ability of *Aa* Y4 to activate TGF- β Rs was measured in phosphor-serine immuno-blots of TGF- β RI immunoprecipitated from OBA9 cells and in cells treated with *Aa* Y4. The incubation of OBA9 cells for 1 hr with *Aa* Y4 markedly increased the phosphorylation of

serine in TGF- β RI approximately 6-fold compared with basal levels (Figs. 2A, 2B). In control experiments, the incubation of OBA9 cells for 1 hr with 10 ng/mL TGF- β 1 also increased the phosphorylation of serine in TGF- β RI, approximately 7-fold compared with basal levels (Figs. 2A, 2B). We then performed an inhibitor assay using SB431542 and siRNA transfection for TGF- β RI. As expected, SB431542 inhibited the *Aa* Y4-enhanced phosphorylation of smad2 in HGECS and OBA9 cells (Figs. 3A, 3B). Therefore, we performed siRNA transfection for TGF- β RI.

We first confirmed that siRNA transfection for TGF- β RI decreased the expression of TGF- β RI at both mRNA and protein levels (Figs. 3C, 3D). Knockdown by siRNA for TGF- β RI attenuated the *Aa* Y4-induced phosphorylation of smad2 in OBA9 cells (Fig. 3E). These results indicated that *Aa* Y4 enhanced the phosphorylation of smad2 through TGF- β RI in OBA9 cells.

Aa Y4 has been shown to induce apoptosis in human gingival epithelial cells (Li *et al.*, 2011; Kang *et al.*, 2012). To test this premise, we exposed OBA9 cells to *Aa* Y4 for various periods. *Aa* Y4 increased the protein level of cleaved caspase-3 in a time-dependent manner, and a maximal effect was observed at 6 hr (Fig. 4A). Furthermore, as shown in Fig. 4 (D, E), TUNEL staining showed that *Aa* Y4 increased the number of apoptotic-positive cells. The pre-treatment with SB431542 and TGF- β RI knockdown by TGF- β RI siRNA decreased the protein levels of cleaved caspase-3 (Figs. 4B, 4C). Consistent with these results, as shown in Fig. 4 (D, E), TUNEL staining showed that TGF- β RI knockdown by TGF- β RI siRNA transfection significantly abrogated the *Aa* Y4-induced increase in the number of apoptotic-positive cells. Next, to address the relationship between smad2 and *Aa* Y4-induced apoptosis in OBA9 cells, we performed siRNA transfection for smad2. As shown in Fig. 4F, we confirmed that siRNA transfection for smad2 decreased the expression of smad2 at the protein levels. Knockdown by siRNA for smad2 apparently attenuated the *Aa* Y4-induced activation of cleaved caspase-3 in OBA9 cells (Fig. 4G). In agreement with this result, TUNEL staining showed that smad2 knockdown by siRNA transfection significantly decreased the *Aa* Y4-increased number of apoptotic-positive cells (Fig. 4H). Taken together, these results demonstrated that *Aa* Y4 induced apoptosis by activating the TGF- β R/sm2 signaling pathway in OBA9 cells.

DISCUSSION

In the present study, we demonstrated, for the first time, that the phosphorylation of smad2 was facilitated through TGF- β RI by the periodontopathic bacteria, *Aa* Y4. We examined the relationship between smad2 and *Escherichia coli* (*E.coli*) and *Aa* strain IDH781 in OBA9 cells. Interestingly, *E.coli* and *Aa* IDH781 also enhanced the phosphorylation of smad2 in OBA9 cells (Appendix Fig., A, B). This result may provide a novel insight into the molecular mechanisms underlying bacterial infection. TGF- β R/sm2 signaling may be associated with many species of bacteria such as *Helicobacter pylori*, *Shigella*, and *E.coli*, in addition to periodontopathic bacteria in the intestinal or gastric epithelium, and may also be positively involved in intestinal or gastric inflammation. Bacterial stimulation in smad2 signaling should be examined in more detail in future studies.

The most important result of the present study was that smad2 was phosphorylated via TGF- β RI by *Aa* stimulation. *Aa* may activate TGF- β R/sm2 signaling in OBA9 cells in 3 ways. The first is an *Aa*-derived TGF- β mimicry molecule. However, it is unlikely that *Aa* binds directly to TGF- β Rs because of their molecular and protein structures. The second is gingival epithelial cell-derived TGF- β . A previous study demonstrated that TGF- β 1 produced by gastric epithelial cells in response to

Figure 2. *Aa* Y4 activated the TGF- β type I receptor. **(A)** Representative Western blotting of *Aa* Y4- and TGF- β 1-stimulated phosphorylation of serine in TGF- β RI and also of total TGF- β RI after the immunoprecipitation (IP) of TGF- β RI. Lane 1, basal; lane 2, *Aa* Y4; lane 3, 10 ng/mL TGF- β 1. **(B)** Densitometric analysis of the *Aa* Y4- or TGF- β 1-stimulated phosphorylation of serine in TGF- β RI. OBA9 cells were incubated for 1 hr with *Aa* Y4 or 10 ng/mL TGF- β 1 as a control. TGF- β RI was immunoprecipitated from cell lysates and subjected to Western blotting analysis of the phosphorylation of serine. Blots were re-probed for total TGF- β RI levels. Results are expressed as a percentage above basal levels in relative densitometric units normalized to total TGF- β RI levels. Band densities were quantified through densitometric scanning of each band with the National Institutes of Health Image J software. Values represent the mean $\pm$ SD of 3 cultures. ** p < .01 values differ significantly (t test).

bacterial infection acted in an autocrine or paracrine manner (Beswick *et al.*, 2011); therefore, TGF- β may be secreted or released from gingival epithelial cells treated with *Aa* and bind to TGF- β Rs to induce the phosphorylation of smad2. The last is *Aa*-dependent TGF- β RI transactivation. Accumulating evidence indicates that there is extensive crosstalk between integrins and TGF- β R signaling. For example, previous studies reported that α 3 β 1 and TGF- β R mediated the phosphorylation of smad2 (KK Kim *et al.*, 2009; Y Kim *et al.*, 2009). Conversely, other studies described an interplay between bacteria and integrin (Blix *et al.*, 1999; Ouhara *et al.*, 2006; Dileepan *et al.*, 2007). Taken together, these findings suggest that smad2 may be phosphorylated by an *Aa*-integrin-TGF- β RI transactivation pathway

Figure 3. *Aa* Y4 induced the phosphorylation of smad2 via the TGF- β type I receptor in OBA9 cells. (A, B) HGEs or OBA9 cells were pre-treated with or without SB431542 (10 μ M) for 30 min and were then exposed to *Aa* Y4 for 1 hr. Phosphorylated (p) and total (t) smad2 levels were analyzed through Western blotting, respectively. (A) HGEs. (B) OBA9 cells. (C, D) OBA9 cells, having been transfected with the negative control (neg) or TGF- β RI siRNA, were cultured for 48 hr in medium B. (C) mRNA levels of TGF- β RI and GAPDH in the cells were analyzed through real-time PCR. (D) Protein levels of TGF- β RI and β -actin were analyzed through Western blotting. (E) OBA9 cells, having been transfected with the negative control or TGF- β RI siRNA, were cultured for 48 hr in medium B and were then exposed to *Aa* Y4 for 1 hr. Phosphorylated (p) and total (t) smad2 levels were analyzed through Western blotting. Band densities were quantified through the densitometric scanning of each band with National Institutes of Health Image J software. Values represent the mean $\pm$ SD of the 3 cultures. ** p < .01 values differ significantly (t test). Similar results were obtained from 3 experiments.

in gingival epithelial cells. This mechanism needs to be elucidated in more detail.

The turnover of the gastrointestinal epithelium is rapid and requires an appropriate balance between the proliferation of progenitor cells and the loss of mature cells. Activation of the

TGF- β R/sm2 cascade is crucially involved in the physiological apoptosis in superficial gastric epithelial cells, which suggests that TGF- β /sm2 signaling may play an important role in the maintenance of homeostasis in the gingival epithelium (Ohgushi *et al.*, 2005). In periodontal tissue, the turnover of the gingival junctional epithelium is also rapid, and the superficial mature cells of the junctional gingival epithelium are thought to be regulated by physiological apoptosis. These findings suggest that the excessive acceleration of TGF- β R/sm2 signaling by microbial pathogens may disrupt the homeostasis of turnover in the gingival epithelium, thereby initiating the onset of periodontitis.

Many microbial pathogens activate the production of pro-inflammatory cytokines and apoptosis-related factors through the toll-like receptor (TLR) pathway. Most studies conducted thus far have indicated a relationship between *Aa*-caused apoptosis and TLRs in gingival epithelial cells (Kato *et al.*, 2013; Park *et al.*, 2014; Shenker *et al.*, 2014). Therefore, the *Aa*-induced phosphorylation of smad2 may occur through a TLR-dependent or -independent pathway.

In this study, it is still unclear which virulence factor from *Aa* induced apoptosis via TGF- β RI-sm2-caspase-3 signaling. CDT from *Aa* is known to induce apoptosis of gingival epithelial cells (Alaoui-El-Azher *et al.*, 2010). However, the other virulence factors may be involved in *Aa* Y4-induced phosphorylation of smad2, because the formalin killed-*Aa* Y4 used in this study does not release CDT. The further study for virulence factors from *Aa* on phosphorylation of smad2 should be necessary.

In summary, the present study provided a novel insight into the bacterial infection system and introduced novel molecular targets in the search for a therapeutic chemical compound that can protect OBA9 cells from bacteria-induced apoptosis. These results may lead to the development of novel preventive or therapeutic interventions against

periodontitis. Future studies are warranted to investigate this in more detail. If bacteria do play a role in TGF- β R/sm2 signaling in various cells, the results of the present study may lead to the mechanisms involved in infectious diseases being elucidated.

Figure 4. Aa Y4 induced apoptosis through the smad2/ TGF- β type I receptor in OBA9 cells. **(A)** OBA9 cells were exposed to Aa Y4 for the indicated times. Cleaved caspase-3 levels and β -actin were analyzed through Western blotting. **(B)** OBA9 cells were pre-treated with or without SB431542 (10 μ M) for 30 min and were then exposed to Aa Y4 for 12 hrs. **(C)** OBA9 cells, having been transfected with the negative control or TGF- β RI siRNA, were cultured for 48 hr in medium B and were then exposed to Aa Y4 for 12 hr. Cleaved caspase-3 levels and β -actin were analyzed through Western blotting. **(D, E)** OBA9 cells, having been transfected with the negative control or TGF- β RI siRNA, were cultured for 48 hr in medium B and were then exposed to Aa Y4 for 24 hr. TUNEL-positive apoptotic cells (red) are shown under each set of conditions, and the graph shows the percentage of TUNEL-positive cells. **(F, G)** OBA9 cells, having been transfected with the negative control or smad2 siRNA, were cultured for 48 hr in medium B and were then exposed to Aa Y4 for 12 hr. **(F)** Total smad2 and β -actin were analyzed through Western blotting. **(G)** Cleaved caspase-3 levels and β -actin were analyzed through Western blotting. **(H)** OBA9 cells, having been transfected with the negative control or TGF- β RI siRNA, were cultured for 48 hr in medium B and were then exposed to Aa Y4 for 24 hr. The graph shows the percentage of TUNEL-positive cells. Band densities were quantified through the densitometric scanning of each band with the National Institutes of Health Image J software. Similar results were obtained from 3 experiments.

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REFERENCES

- Abuhussein H, Bashutski JD, Dabiri D, Halubai S, Layher M, Klausner C, *et al.* (2013). The role of factors associated with apoptosis in assessing periodontal disease status. *J Periodontol* 85:1086-1095.
- Alaoui-El-Azher M, Mans JJ, Baker HV, Chen C, Progulske-Fox A, Lamont RJ, *et al.* (2010). Role of the ATM-checkpoint kinase 2 pathway in CDT-mediated apoptosis of gingival epithelial cells. *PLoS One* 5:e11714.
- Beswick EJ, Pinchuk IV, Earley RB, Schmitt DA, Reyes VE (2011). Role of gastric epithelial cell-derived transforming growth factor beta in reduced CD4+ T cell proliferation and development of regulatory T cells during *Helicobacter pylori* infection. *Infect Immun* 79:2737-2745.
- Blix IJ, Helgeland K, Kähler H, Lyberg T (1999). LPS from *Actinobacillus actinomycetemcomitans* and the expression of beta2 integrins and L-selectin in an ex vivo human whole blood system. *Eur J Oral Sci* 107:14-20.
- Derynck R, Zhang YE (2003). Smad-dependent and Smad-independent pathways in TGF- β family signalling. *Nature* 425:577-584.
- Dickinson BC, Moffatt CE, Hagerty D, Whitmore SE, Brown TA, Graves DT, *et al.* (2011). Interaction of oral bacteria with gingival epithelial cell multilayers. *Mol Oral Microbiol* 26:210-220.
- Dileepan T, Kachlany SC, Balashova NV, Patel J, Maheswaran SK (2007). Human CD18 is the functional receptor for *Aggregatibacter actinomycetemcomitans* leukotoxin. *Infect Immun* 75:4851-4856.
- Fujita T, Ashikaga A, Shiba H, Uchida Y, Hirano C, Iwata T, *et al.* (2006). Regulation of IL-8 by Irsogladine maleate is involved in abolishment of *Actinobacillus actinomycetemcomitans*-induced reduction of gap-junctional intercellular communication. *Cytokine* 34:271-277.
- Fujita T, Kishimoto A, Shiba H, Hayashida K, Kajiya M, Uchida Y, *et al.* (2010). Irsogladine maleate regulates neutrophil migration and E-cadherin expression in gingival epithelium stimulated by *Aggregatibacter actinomycetemcomitans*. *Biochem Pharmacol* 79:1496-1505.

- Fujita T, Alotaibi M, Kitase Y, Kota Y, Ouhara K, Kurihara H, *et al.* (2012). Smad2 is involved in the apoptosis of murine gingival junctional epithelium associated with inhibition of Bcl-2. *Arch Oral Biol* 57:1567-1573.
- Höglund Åberg C, Antonoglou G, Haubek D, Kwamin F, Claesson R, Johansson A (2013). Cytolethal distending toxin in isolates of *Aggregatibacter actinomycetemcomitans* from Ghanaian adolescents and association with serotype and disease progression. *PLoS One* 8:e65781.
- Kachlany SC (2010). *Aggregatibacter actinomycetemcomitans* leukotoxin: from threat to therapy. *J Dent Res* 89:561-570.
- Kang J, de Brito Bezerra B, Pacios S, Andriankaja O, Li Y, Tsiagbe V, *et al.* (2012). *Aggregatibacter actinomycetemcomitans* infection enhances apoptosis in vivo through a caspase-3-dependent mechanism in experimental periodontitis. *Infect Immun* 80:2247-2256.
- Kato S, Nakashima K, Inoue M, Tomioka J, Nonaka K, Nishihara T, *et al.* (2000). Human epithelial cell death caused by *Actinobacillus actinomycetemcomitans* infection. *J Med Microbiol* 49:739-745.
- Kato S, Nakashima K, Nagasawa T, Abiko Y, Furuichi Y (2013). Involvement of Toll-like receptor 2 in apoptosis of *Aggregatibacter actinomycetemcomitans*-infected THP-1 cells. *J Microbiol Immunol Infect* 46:164-170.
- Kim KK, Wei Y, Szekeres C, Kugler MC, Wolters PJ, Hill ML, *et al.* (2009). Epithelial cell alpha3beta1 integrin links beta-catenin and Smad signaling to promote myofibroblast formation and pulmonary fibrosis. *J Clin Invest* 119:213-224.
- Kim Y, Kugler MC, Wei Y, Kim KK, Li X, Brumwell AN, *et al.* (2009). Integrin alpha3beta1-dependent beta-catenin phosphorylation links epithelial Smad signaling to cell contacts. *J Cell Biol* 184:309-322.
- Kusumoto Y, Hirano H, Saitoh K, Yamada S, Takedachi M, Nozaki T, *et al.* (2004). Human gingival epithelial cells produce chemotactic factors interleukin-8 and monocyte chemoattractant protein-1 after stimulation with *Porphyromonas gingivalis* via Toll-like receptor 2. *J Periodontol* 75:370-379.
- Lee JH, Wan XH, Song J, Kang JJ, Chung WS, Lee EH, *et al.* (2002). TGF-beta-induced apoptosis and reduction of Bcl-2 in human lens epithelial cells in vitro. *Curr Eye Res* 25:147-153.
- Li Y, Shibata Y, Zhang L, Kuboyama N, Abiko Y (2011). Periodontal pathogen *Aggregatibacter actinomycetemcomitans* LPS induces mitochondria-dependent-apoptosis in human placental trophoblasts. *Placenta* 32:11-19.
- Mithani SK, Balch GC, Shiou SR, Whitehead RH, Datta PK, Beauchamp RD (2004). Smad3 has a critical role in TGF-beta-mediated growth inhibition and apoptosis in colonic epithelial cells. *J Surg Res* 117:296-305.
- Ohgushi M, Kuroki S, Fukamachi H, O'Reilly LA, Kuida K, Strasser A, *et al.* (2005). Transforming growth factor beta-dependent sequential activation of Smad, Bim, and caspase-9 mediates physiological apoptosis in gastric epithelial cells. *Mol Cell Biol* 25:10017-10028.
- Ouhara K, Komatsuzawa H, Shiba H, Uchida Y, Kawai T, Sayama K, *et al.* (2006). *Actinobacillus actinomycetemcomitans* outer membrane protein 100 triggers innate immunity and production of beta-defensin and the 18-kilodalton cationic antimicrobial protein through the fibronectin-integrin pathway in human gingival epithelial cells. *Infect Immun* 74:5211-5220.
- Park SR, Kim DJ, Han SH, Kang MJ, Lee JY, Jeong YJ, *et al.* (2014). Diverse Toll-like receptors mediate cytokine production by *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans* in macrophages. *Infect Immun* 82:1914-1920.
- Ramjaun AR, Tomlinson S, Eddaoudi A, Downward J (2007). Upregulation of two BH3-only proteins, Bmf and Bim, during TGF beta-induced apoptosis. *Oncogene* 26:970-981.
- Rogers JE, Li F, Coatney DD, Rossa C, Bronson P, Krieder JM, *et al.* (2007). *Actinobacillus actinomycetemcomitans* lipopolysaccharide-mediated experimental bone loss model for aggressive periodontitis. *J Periodontol* 78:550-558.
- Schreiner HC, Sinatra K, Kaplan JB, Furgang D, Kachlany SC, Planet PJ, *et al.* (2003). Tight-adherence genes of *Actinobacillus actinomycetemcomitans* are required for virulence in a rat model. *Proc Natl Acad Sci USA* 100:7295-7300.
- Schroeder HE, Listgarten MA (1997). The gingival tissues: the architecture of periodontal protection. *Periodontol* 2000 13:91-120.
- Shenker BJ, Walker LP, Zekavat A, Dlakić M, Boesze-Battaglia K (2014). Blockade of the PI-3K signaling pathway by the *Aggregatibacter actinomycetemcomitans* cytolethal distending toxin induces macrophages to synthesize and secrete pro-inflammatory cytokines. *Cell Microbiol* 16:1391-1404.
- Tonetti MS, Cortellini D, Lang NP (1998). In situ detection of apoptosis at sites of chronic bacterially induced inflammation in human gingiva. *Infect Immun* 66:5190-5195.
- Uchida Y, Shiba H, Komatsuzawa H, Hirono C, Ashikaga A, Fujita T, *et al.* (2005). Irsogladine maleate influences the response of gap junctional intercellular communication and IL-8 of human gingival epithelial cells following periodontopathogenic bacterial challenge. *Biochem Biophys Res Commun* 333:502-507.
- Wildev GM, Patil S, Howe PH (2003). Smad3 potentiates transforming growth factor beta (TGFbeta)-induced apoptosis and expression of the BH3-only protein Bim in WEHI 231 B lymphocytes. *J Biol Chem* 278:18069-18077.
- Wilson M, Henderson B (1995). Virulence factors of *Actinobacillus actinomycetemcomitans* relevant to the pathogenesis of inflammatory periodontal diseases. *FEMS Microbiol Rev* 17:365-379.
- Yang J, Wahdan-Alaswad R, Danielpour D (2009). Critical role of Smad2 in tumor suppression and transforming growth factor-beta-induced apoptosis of prostate epithelial cells. *Cancer Res* 69:2185-2190.