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Signaling transduction analysis in gingival epithelial cells after infection with *Aggregatibacter actinomycetemcomitans*

J.E. Umeda[1], D.R. Demuth[2], E.S. Ando[1], M. Faveri[3] and M.P.A. Mayer[1]

1 Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil
2 School of Dentistry, University of Louisville, Louisville, KY, USA
3 Department of Periodontology, Dental Research Division, Guarulhos University, Guarulhos, Brazil

Correspondence: Marcia Pinto Alves Mayer, Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo Ed. Biomédicas II Av. Lineu Prestes, 1374 São Paulo, SP, 05508-900, Brazil Tel.: +55 11 3091 7348; fax: +55 11 3091 7354;
E-mail: mpamayer@usp.br

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SUMMARY

Periodontal diseases result from the interaction of bacterial pathogens with the host's gingival tissue. Gingival epithelial cells are constantly challenged by microbial cells and respond by altering their transcription profiles, inducing the production of inflammatory mediators. Different transcription profiles are induced by oral bacteria and little is known about how the gingival epithelium responds after interaction with the periodontopathogenic organism *Aggregatibacter actinomycetemcomitans*. In the present study, we examined the transcription of genes involved in signaling transduction pathways in gingival epithelial cells exposed to viable *A. actinomycetemcomitans*. Immortalized gingival epithelial cells (OBA-9) were infected with *A. actinomycetemcomitans* JP2 for 24 h and the transcription profile of genes encoding human signal transduction pathways was determined. Functional analysis of inflammatory mediators positively transcribed was performed by ELISA in culture supernatant and in gingival tissues. Fifteen of 84 genes on the array were over-expressed ($P < 0.01$) after 24 h of infection with viable *A. actinomycetemcomitans*. Over-expressed genes included those implicated in tissue remodeling and bone resorption, such as CSF2, genes encoding components of the LDL pathway, nuclear factor-κB-dependent genes and other cytokines. The ELISA data confirmed that granulocyte–macrophage colony-stimulating factor/colony-stimulating factor 2, tumor necrosis factor-α and intercellular adhesion molecule-1 were highly expressed by infected gingival cells when compared with control non-infected cells, and presented higher concentrations in tissues from patients with aggressive and chronic periodontitis than in tissues from healthy controls. The induction in epithelial cells of factors such as the pro-inflammatory cytokine CSF2, which is involved in osteoclastogenesis, may help to explain the outcomes of *A. actinomycetemcomitans* infection.

INTRODUCTION

Although the oral cavity harbors over 700 bacterial species (Aas *et al.*, 2005), only a small subset of species is associated with the destruction of periodontal tissues. *Aggregatibacter actinomycetemcomitans* is strongly implicated in the etiology of aggressive periodontitis (Slots *et al.*, 1980) but is also

associated with systemic infections (Muhle *et al.*, 1979; Paturel *et al.*, 2004). *Aggregatibacter actinomycetemcomitans* is part of the HACEK group, a group of gram-negative, fastidious, slow-growing oropharyngeal bacilli, which contributes to infective endocarditis in humans (Paturel *et al.*, 2004). The main niche for this species is the oral mucosa, particularly the gingival sulcus/pocket. The epithelial cells act as sensors during microbial infections (Dale, 2002) through the recognition of microbe-associated molecular patterns by Toll-like receptors and other pattern recognition receptors (Kikkert *et al.*, 2007). Following the receptor–ligand interaction, signal transduction is initiated (Laube *et al.*, 2008) inducing and maintaining an inflammatory response in the periodontium (Silva *et al.*, 2008). Although the host response is intended to fight off the bacterial infection associated with periodontitis, the elevated levels of inflammatory cytokines may result in an exacerbated host response, leading to production of matrix metalloproteinases, osteoclast activation and further bone resorption (Fukushima *et al.*, 2005).

Different pathogens induce a variety of epithelial cell responses (Milward *et al.*, 2007; Shan *et al.*, 2007), and little is known about the gingival epithelial cell response to *A. actinomycetemcomitans*. In addition to adhesion to host cells, *A. actinomycetemcomitans* can invade non-phagocytic cells such as epithelial cells (Meyer *et al.*, 1996), and interact with intracellular receptors (Shan *et al.*, 2007). Hence, pathogenic bacteria manipulate the host response to improve their survival by activating or inhibiting different signaling pathways. The increase in our knowledge of the strategies used by the pathogens during the infection may result in the development of new therapeutic strategies in the treatment of periodontal diseases, such as pharmacological products antagonizing the effect induced by bacteria and their products (Shan *et al.*, 2007; Yoshida & Yoshikawa, 2008). To contribute to the understanding of the inflammatory scenario in the periodontium seen in aggressive periodontitis, this study compared the transcription of genes encoding components of signal transduction pathways in *A. actinomycetemcomitans*-infected and uninfected gingival epithelial cells. In addition, the induction of positively regulated genes was confirmed by determining the concentration of these factors by enzyme-linked immunosorbent assay (ELISA) in the supernatant of *A. actinomycetemcomitans*-infected cells as well as in the gingival tissues of patients with aggressive and chronic periodontitis and in healthy controls.

METHODS

Bacterial strain and culture conditions

Aggregatibacter actinomycetemcomitans strain JP2 was used to infect oral epithelial cells. Bacteria were grown in 5% CO_2 (microaerophilic conditions) at 37°C in brain–heart infusion broth (BHI; Difco, Sparks, MD) supplemented with 40 mg $NaHCO_3$ l^{-1}.

Epithelial cell culture

Immortalized normal gingival epithelial cell line (OBA-9) was grown in serum-free keratinocyte growth medium (KSFM; Invitrogen, Carlsbad, CA) containing insulin, epidermal growth factor and fibroblast growth factor (Invitrogen), supplemented with 100 μg ml^{-1} streptomycin and 100 U ml^{-1} penicillin (Sigma, St Louis, MO) and cultured at 37°C in 5% CO_2 (TC 2123; Shell Lab, Cornelius, OR).

Adhesion and invasion assays

Gingival epithelial cells (OBA-9) ($\sim 2 \times 10^5$ cells per well) were inoculated in 24-well tissue culture plates (Corning Inc., Corning, NY) and incubated to reach a semi-confluent monolayer ($\sim 3 \times 10^5$ OBA-9 cells per well) in KSFM. Before infection, the wells were washed thrice with phosphate-buffered saline (pH 7.5, 0.8% NaCl). Overnight bacterial cultures were inoculated in BHI broth to reach the exponential growth phase/mid log, harvested by centrifugation, and resuspended in antibiotic-free KSFM medium. The bacterial colony-forming unit levels were confirmed by viable counting. OBA-9 cells in semi-confluent monolayers were inoculated with bacteria in antibiotic-free KSFM at a multiplicity of infection 100 : 1 ($\sim 3 \times 10^7$ bacteria per well). After incubation for 2, 12 and 24 h, the wells were washed thrice and treated with 0.25% trypsin/EDTA. The number of viable bacterial cells adhesive to cells per well was estimated by culture in BHI agar plates. For the invasion assay, OBA-9 cells were co-cultured with bacterial cells for 2, 12 and 24 h, unattached bacteria were removed by washings, and extracellular

bacteria were killed by incubation with gentamicin (100 μg ml^{-1}) (Sigma) for 60 min. After washing, internalized bacteria were released by epithelial cell lysis with 0.5% Triton X-100 (Sigma) (Meyer *et al.*, 1996) and the number of viable bacterial cells per well was determined. The experiments were performed in triplicate in three independent assays.

Transcription analysis of genes involved in cell signaling pathway

Similar assays to the adhesion assay were performed for gene transcription analysis after 24 h of co-culture with *A. actinomycetemcomitans*, except for the trypsin treatment. The transcription of 84 genes was evaluated in cells infected with *A. actinomycetemcomitans* and compared with control non-infected cells. Total RNA was obtained using Trizol (Invitrogen), according to the manufacturer's instructions. After chloroform extraction, RNA was precipitated with isopropanol and washed with 70% ethanol. RNA quantity and integrity were determined by spectrophotometer (ND-1000; Nanodrop, Wilmington, DE). Contaminating genomic DNA was removed by DNase digestion. First-strand synthesis was performed on 1 μg RNA using an RT_2CR Array First Strand kit (Bioscience Corporation, Frederick, MD). Gene expression was determined by real-time polymerase chain reaction using the Human Signal Transduction Pathway Finder (Bioscience Corporation, Frederick, MD). Experimental and control samples were analyzed in triplicate.

Expression profiles of the target genes were measured relative to the mean cycle threshold (C_t) values of the five different calibrator genes (*b2m*, *hprt1*, *rpl13a*, *gapdh* and *actb*) using the $\Delta\Delta C_t$ method. For statistical comparison of the control and experimental groups, Student's *t*-test was performed using mean C_t values derived from the triplicate samples. Significant difference in gene expression was considered when $P < 0.01$ at greater than or equal to a fourfold change in expression.

Detection of apoptosis

Interaction between bacteria and eukaryotic cells was performed as described for the adhesion assay. After 24 h of interaction, apoptosis was evaluated by using the annexin V assay as described by the manufacturer (FITC AnnexinV apoptosis detection kit; BD Pharmigen, San Jose, CA) (Vermes *et al.*, 1995). Cells stained with FITC-conjugated annexin V and propidium iodide were analyzed by flow cytometry (Guava Easy cyteFlow Cytometer; Millipore, Billerica, MA). Ten thousand events were analyzed in FL-1 (FITC) and FL-2 (propidium iodide) channels. As a positive control, camptothecin (6 μM final concentration) (Sigma) was added to the OBA-9 control cells 4 h before reading. Cells with the annexin V-positive, propidium iodide-negative phenotype were defined as early apoptotic cells.

Quantification of colony-stimulating factor 2/granulocyte–macrophage colony-stimulating factor, tumor necrosis factor-α and intercellular adhesion molecule-1 in the supernatant of infected gingival epithelial cells and in gingival tissues

The supernatants of gingival epithelial cells (OBA-9) exposed to bacteria cells for 24 h; as well as control cells, and of homogenized gingival tissues were evaluated. This study was approved previously by the ethical committees for human research (University of São Paulo, and University of Guarulhos). The protocol was explained to each participant and signed informed consents were obtained. Gingival tissue samples were obtained from surgery from patients with different periodontal conditions selected from the population referred to the periodontal clinic of the Guarulhos University: periodontally healthy ($n = 12$), aggressive periodontitis ($n = 10$) and chronic periodontitis ($n = 11$). Subjects were diagnosed based on the periodontal classification of the American Academy of Periodontology (Armitage, 1999), and followed the criteria as previously described (Faveri *et al.*, 2009). After surgery, 100 mg of gingival tissue was added to 500 μl lysis buffer [phosphate-buffered saline (1×)/0.05% Tween-20/1 mM protease inhibitor; Sigma] and homogenized with a Dounce Glass homogenizer. Insoluble debris was removed by centrifugation. The levels of colony-stimulating factor 2 (CSF2)/granulocyte–macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α) and intercellular adhesion molecule 1 (ICAM-1) were determined in the soluble fraction of gingival tissue samples and in the cell supernatants by ELISA, according to the manufacturer's protocols [CSF2/GM-CSF and TNF-α (Peprotech Inc.,

Rocky Hill, NJ) and ICAM-1 (R&D Systems, Minneapolis, MN)]. The absorbance at 450 nm was read using a microplate reader (Bio-Rad, Hercules, CA; model 680) with a wavelength correction set at 550 nm. To access the levels of colonizing *A. actinomycetemcomitans* in each studied site, subgingival biofilm was collected from areas adjacent to each excised gingival site and levels of bacteria were determined using checkerboard DNA–DNA hybridization (Faveri *et al.*, 2009). Student's *t*-test was used to determine differences in inflammatory mediator levels secreted into supernatants of control cells and infected gingival epithelial cells (OBA-9). Kruskal–Wallis test was used to compare the levels of inflammatory mediators in gingival tissues of subjects with healthy gingiva, or with aggressive or chronic periodontitis. Significant differences were considered when $P < 0.05$.

RESULTS

The adhesion and invasion properties of *A. actinomycetemcomitans* JP2 were established at different periods of interaction to select the appropriate time for signaling transduction analysis in OBA-9 cells. Adhesion was more efficient after 2 h of infection and decreased after 24 h of interaction, whereas invasion increased from 2 to 12 h, and remained stable until 24 h (Fig. 1).

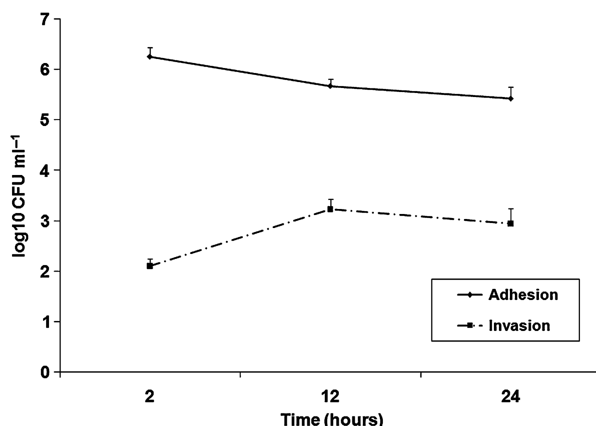

Figure 1 Number of bacterial cells expressed as $\log_{10}$ colony-forming units (CFU) ml^{-1}, determined in adhesion and invasion assays for gingival epithelial cells (OBA-9) after 2, 12 and 24 h of co-culture with *Aggregatibacter actinomycetemcomitans* JP2. Multiplicity of infection (MOI) 100 : 1.

Gene expression assay

The transcription profile in OBA-9 cells after infection with *A. actinomycetemcomitans* for 24 h indicated upregulation of 15 genes among 84 target genes of the human signal transduction pathway (Table 1) with statistically significant differences ($P < 0.01$). The gene *csf2*, encoding CSF2/GM-CSF, was the most upregulated gene (170-fold change) as shown in Table 1. The genes encoding TNF-α and early growth response-1 (Egr-1) were the second (28.93 change) and third (14.08 change) most positively regulated genes in infected cells.

Apoptosis assay

To verify the apoptosis after 24 h of interaction, Annexin V staining was performed. As shown in Fig. 2, *A. actinomycetemcomitans* JP2 did not lead to apoptosis when compared with negative control.

Enzyme-linked immunosorbent assay

The concentrations of GM-CSF/CSF2, TNF-α and ICAM-1 were evaluated by ELISA, to confirm whether factors encoded by genes positively transcribed in the array were expressed at increased levels in culture supernatant by the infected epithelial cells. The concentration of GM-CSF/CSF2 reached high levels in infected cells, and was not detected in the supernatant of control cells (Fig. 3A). High levels of TNF-α were observed in the JP2-infected cells, with significant difference than control (Fig. 3B). Infected gingival epithelial cells also expressed higher levels of the adhesion molecule ICAM-1 than controls (Fig. 3C). Significant differences in the levels for GM-CSF/CSF2, TNF-α and ICAM-1 were also shown in gingival tissue from periodontitis patients when compared with tissues of periodontally health patients, but not between chronic and aggressive periodontitis (Fig. 4).

Aggregatibacter actinomycetemcomitans was detected by DNA–DNA hybridization in levels equivalent to or higher than 10^5 cells per sample in five of ten subgingival samples taken from areas adjacent to the excised gingival tissue in patients with aggressive periodontitis, whereas these levels were detected in three of eleven chronic periodontitis sites and in none of the sites in healthy subjects. However, there was no correlation between subgingival levels of

Table 1 Genes in the signaling transduction pathway upregulated in *Aggregatibacter actinomycetemcomitans*-infected OBA-9 gingival epithelial cells, in comparison with non-infected control cells

Symbol	Pathway	Gene	GenBank	Fold changes	*P*-value
BIRC3	NF-κB	Baculoviral IAP repeat-containing 3	NM_001165	5.92	0.000022
CCL2	LDL	Chemokine (C-C motif) ligand 2	NM_002982	12.18	0.000003
CCL20	NF-κB	Chemokine (C-C motif) ligand 20	NM_004591	6.49	0.0012
CDKN1A	TGF-β/p53/Androgen	Cyclin-dependent kinase inhibitor 1A	NM_000389	4.88	0.0015
CSF2	LDL/calcium and protein quinase C/NF-κB (Zhang & Rudney, 2011)	Colony stimulating factor 2	NM_000758	170.04	0.00002
EGR1	Mitogenic/CREB/PhospholipaseC	Early growth response 1	NM_001964	14.08	0.00066
FOS	Mitogenic/Stress/PhospholipaseC/ JNK/p38 (Zhang & Rudney, 2011)	V-fos FBJ murine osteosarcoma viral oncogene homolog	NM_005252	4.47	0.004
GADD45A	p53	Growth arrest and DNA-damage-inducible, alpha	NM_001924	8.00	0.00058
GREB1	Estrogen	GREB-1 protein	NM_014668	10.13	0.0088
ICAM1	NF-κB/Phospholipase C	Intercellular adhesion molecule 1 (CD54), human rhiono virus receptor	NM_000201	7.67	0.00003
IL1A	NF-κB	Interleukin 1, alpha	NM_000575	4.01	0.0009
IRF1	JAK-STAT/IRF (Zhang & Rudney, 2011)	Interferon regulatory factor 1	NM_002198	5.39	0.004
JUN	Mitogenic/WNT/PI3 kinase-AKT/Calcium and protein kinase C/PhospholipaseC JNK/p38 (Zhang & Rudney, 2011)	Jun oncogene	NM_002228	7.16	0.00023
TNF	NF-κB	Tumor necrosis factor (TNF superfamily, member 2)	NM_000594	28.93	0.0097
VEGFA	WNT	Vascular endothelial growth factor A	NM_003376	4.59	0.000246

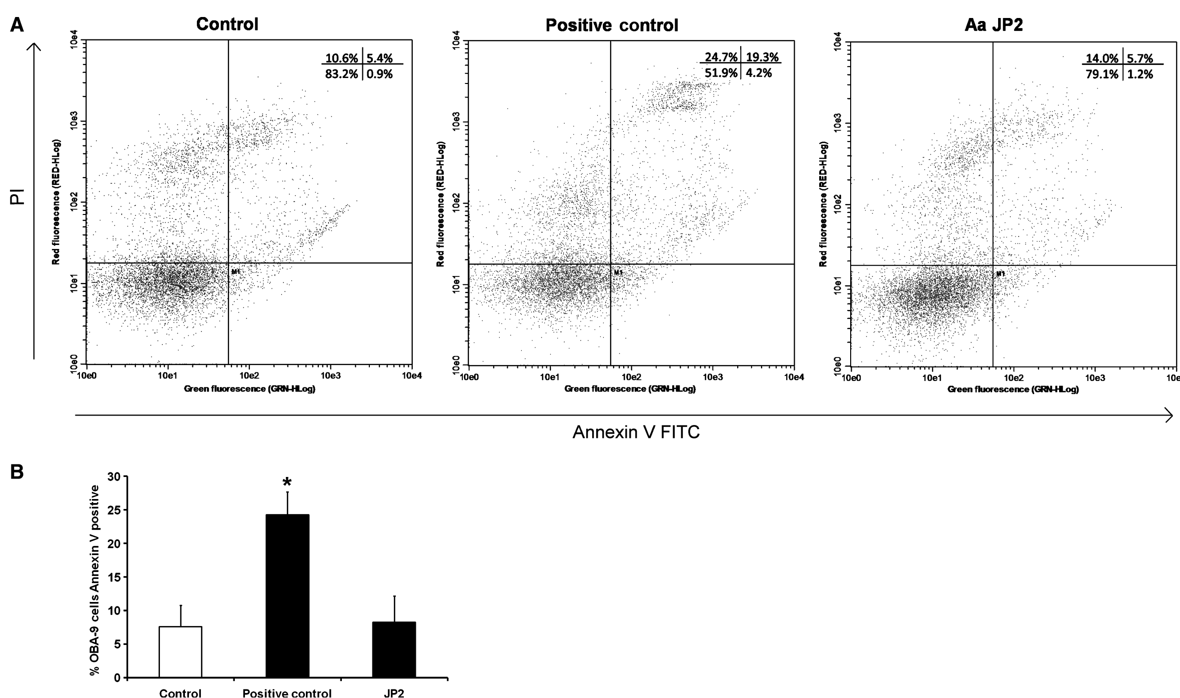

Figure 2 Detection of apoptosis after 24 h of interaction between OBA-9 cells and *Aggregatibacter actinomycetemcomitans* JP2 at multiplicity of infection (MOI) 100 : 1, using annexin V-FITC/PI staining and flow cytometry. (A) Dual parameter represented by dot plots, and the percentage of living, dead, late apoptotic and early apoptotic cells. (B) Percentage of annexin V-positive cells, considering 100% of viability in negative control viable cells. Positive control was OBA-9 cells with camptothecin in a final concentration of 6 μM. *Statistically significant when compared with negative control (Control), when $P < 0.05$ (analysis of variance/Tukey).

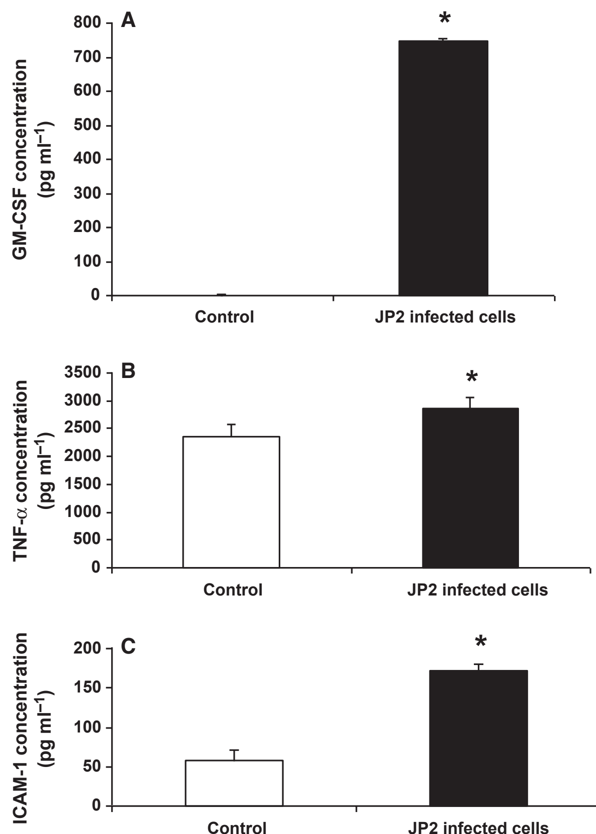

Figure 3 Inflammatory mediator levels measured by ELISA in the supernatant of OBA-9 cells culture-infected for 24 h with 3×10^7 colony-forming units (CFU) ml^{-1} *Aggregatibacter actinomycetemcomitans* JP2. Control, non-infected cells. (A) Granulocyte–macrophage colony-stimulating factor (GM-CSF); (B) tumor necrosis factor-α (TNF-α) and (C) intercellular adhesion molecule 1 (ICAM-1). Student's *t*-test; $^*P < 0.05$).

A. actinomycetemcomitans and of the levels of GM-CSF, TNF-α or ICAM-1 in the adjacent gingival tissues (Spearman Rank Correlation, $P > 0.05$).

DISCUSSION

Data presented here provide evidence on the transcript changes of gingival epithelial cells promoted by infection with *A. actinomycetemcomitans*, identifying a variety of genes and pathways involved in signaling recruitment and activation of immune system cells, along with genes important for cellular and tissue protection from bacteria and other cellular insults. The bacterial challenge consisted not only of bacterial products, but adherent and internalized bacteria were also found, indicating that membrane, as well as intracellular receptors (Nods), were possibly activated during *A. actinomycetemcomitans* infection, as previously suggested (Stathopoulou *et al.*, 2010). Although oral epithelial cells may respond to a bacterial stimulus after a short period of contact, the cascade of transcription increases within time and other factors inducing stress, such as media changes, may result in differential transcription (Milward *et al.*, 2007). Hence, a longer exposure (24-h infection) was chosen for the analysis, which may better represent the conditions occurring *in vivo* during chronic diseases, with epithelial cell multiplication and feedback mechanisms.

Fifteen genes belonging to signaling transduction pathways were upregulated in gingival epithelial cells infected with *A. actinomycetemcomitans*. Our data suggest that interaction with viable adhesive and invasive *A. actinomycetemcomitans* activates several pathways, since the upregulated genes were involved in mitogen-activated protein kinase signaling (*egr1*, *fos* and *jun*), nuclear factor-κB (NF-κB) activation, increase of cytoplasmic calcium concentration and activation of protein kinase C (*csf2/gm-csf*, *fos*, *jun*), activation of phospholipase C (*egr1*, *fos*, *icam-1*, *jun*), LDL (*ccl2* and *csf2*) and p53 (*cdkn1a*, *gadd45a*) pathways in OBA-9 cells. On the other hand, except for *jun*, genes such as *bcl2*, *ccnd1*, *fn1* and *mmp7*, and *myc*, involved in the phosphoinositol-3 kinase pathway were not induced by *A. actinomycetemcomitans* infection. A recent study has shown activation of the FAK–phosphoinositol-3 kinase or Rho-GTPase signaling cascade by *A. actinomycetemcomitans* Omp29, resulting in F-actin rearrangement, which, in turn, can promote bacterial internalization (Kajiya *et al.*, 2011), moreover, CdtB possess phosphatase activity, acting as of phosphatidylinositol 3,4,5-triphosphate phosphatase (Shenker *et al.*, 2007).

The gene for GM-CSF (*csf2* or *gm-csf*) was the most upregulated gene (170.04 times) in gingival epithelial cells infected by *A. actinomycetemcomitans*. ELISA data confirmed the high expression of GM-CSF in infected gingival epithelial cells and in gingival tissues of patients with aggressive periodontitis. Oral epithelial cells exposed to other bacteria, such as *Fusobacterium nucleatum*, had also shown a significant increase in the expression of *CSF2* (17.96 times) (Zhang & Rudney, 2011), although not so drastic as seen for *A. actinomycetemcomitans*-infected cells. CSF2/GM-CSF plays an important role in survival, proliferation and differentiation of neu-

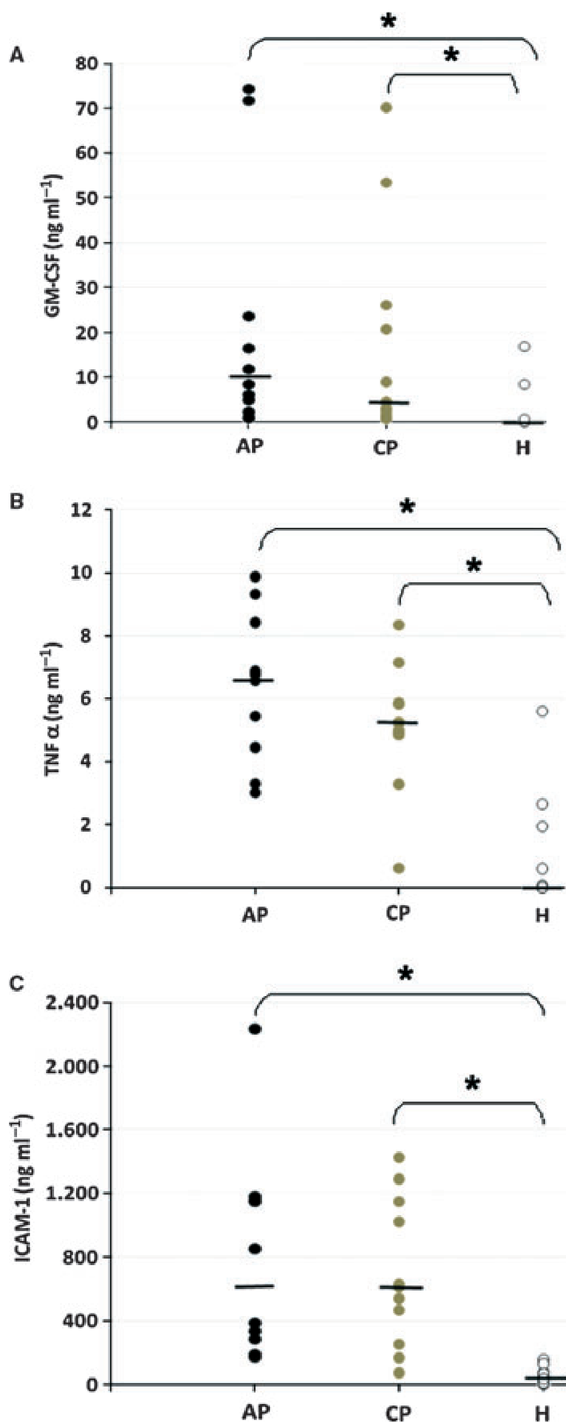

Figure 4 Inflammatory mediator levels measured by ELISA in the gingival tissue samples obtained from subjects with different periodontal conditions: healthy (H) (n = 12), aggressive periodontitis (AP) (n = 10) and chronic periodontitis (CP) (n = 11). (A) Granulocyte–macrophage colony-stimulating factor (GM-CSF); (B) tumor necrosis factor-α (TNF-α) and (C) intercellular adhesion molecule 1 (ICAM-1). (Kruskal–Wallis; $^{*}P < 0.05$). Bars represent median of individual groups.

trophils and macrophages (Hamilton, 2008). However, this factor has also been identified as a key target of NF-κB, and mediates osteoclastic bone destruction (Park *et al.*, 2007). It is expressed at higher levels at sites of inflammation, such as in arthritis (Nomura *et al.*, 2008), and in human periodontal ligament cells after orthodontic tooth movement (MacDonald *et al.*, 1986).

Expression of GM-CSF is linked to the production of pro-inflammatory cytokines TNF-α and interleukin-1 (IL-1; Hamilton, 2008). Both TNF-α and IL-1 were upregulated in *A. actinomycetemcomitans*-infected epithelial cells (28.93-fold change and 4.01-fold change, respectively) and previous data revealed that both are significantly elevated in periodontitis (Fukushima *et al.*, 2005). IL-1 is the most potent mediator of bone resorption in vertebrates (Jimi *et al.*, 1996) and promotes osteoclast formation by regulating RANKL/OPG expression (Fukushima *et al.*, 2005). The TNF-α contributes to the pathogenicity of rheumatoid arthritis, periodontitis and other infections (Feldmann *et al.*, 2001; Feldmann & Maini, 2003) by inducing pro-inflammatory chemoattractant cytokine cascades (O' Hara *et al.*, 2009). The TNF-α also activates the signaling cascades required for NF-κB activation (Yamamoto & Takeda, 2008). Our data indicated that genes encoding factors induced by NF-κB activation such as CCL20, IL-1α, ICAM1 and BIRC3 were also upregulated in *A. actinomycetemcomitans*-infected cells. Other evidence showing NF-κB activation in periodontal tissues came from studies showing that p50 and p65 transcription factors, which are components of the NF-κB complex, exhibited increased activity beneath periodontal lesions (Ambili *et al.*, 2005). Furthermore, elevated NF-κB activity has been demonstrated in the pathogenesis of chronic inflammatory diseases associated with periodontitis and atherosclerosis (Nichols *et al.*, 2001). Activation of NF-κB influences bone resorption and osteoclast differentiation (Park *et al.*, 2007) although it is involved in both protective and destructive responses (Spehlmann & Eckmann, 2009).

Previous data have shown that interaction of epithelial cells with live *A. actinomycetemcomitans* for 6 h resulted in the expression of IL-8 and ICAM-1 (Huang *et al.*, 1998). Infection with *Porphyromonas gingivalis* results also in immediate upregulation of IL-8 mRNA but downregulation occurs simultaneously and is enhanced by the continuous presence of large

numbers of bacteria (Huang *et al.*, 2004). Hence, the lack of an upregulation of IL-8 promoted by *A. actinomycetemcomitans* infection in the present study may be because of the longer exposure to live bacterial cells (24 h).

Our data indicated that transcription of IL-1α, CCL20, GM-CSF, BIRC3 and TNF-α was induced in epithelial cells infected with *A. actinomycetemcomitans*. The same factors were also induced after interaction with *P. gingivalis* and *F. nucleatum* (Milward *et al.*, 2007). However, infection with *A. actinomycetemcomitans* induced the expression of the ICAM-1, which was not reported for *P. gingivalis* and *F. nucleatum* (Milward *et al.*, 2007). The elevated levels of ICAM-1 after interaction of epithelial cells with *A. actinomycetemcomitans* were confirmed in the cell supernatants, as also recently shown (Shimada *et al.*, 2008). The production of ICAM-1 is also induced by TNF-α (Tancharoen *et al.*, 2008) and its levels were associated with the severity of cardiovascular diseases (Lawson & Wolf, 2009). This adhesion molecule is involved in the transendothelial migration of leukocytes to sites of inflammation, in interactions between antigen-presenting cells and T cells, and it may play a direct role in osteoclast formation and function (Bloemen *et al.*, 2009). Genes known to encode the transcription factors Egr-1, FOS and Jun were also positively transcribed in infected cells. Egr-1, was the third most upregulated gene (14.08-fold change) in infected cells. It should be noted that its expression was also induced in *Helicobacter pylori*-infected gastric and colonic cell lines (Abdel-Latif *et al.*, 2004) and it is involved in the activation of NF-κB in certain carcinomas (Wang *et al.*, 2009). Egr-1 plays a role in the induction of the epithelial cell suppressors transforming growth factor-β1 and p53, which promote apoptosis (Baron *et al.*, 2006). In addition, the cell cycle inhibitor p21 (CIP1 or CDKN1A), which is regulated by Egr-1 through direct induction of transforming growth factor-β1 (Polyak *et al.*, 1994), was also upregulated in *A. actinomycetemcomitans*-infected cells.

Factors related to growth inhibition such as *gadd45* (growth arrest and DNA damage-inducible) and p21 (encoded by *cdkn1a* gene) were positively regulated (eightfold change and 4.88-fold change, respectively) in *A. actinomycetemcomitans*-infected cells. Gadd45α seems to be produced during stress stimuli and could lead to apoptosis. However, it is also involved in cellular survival, cell cycle arrest and DNA repair (Liebermann & Hoffman, 2008). In hematopoietic cells exposed to UV radiation, Gadd45α cooperate to promote cell survival by two distinct signaling pathways involving activation of a novel Gadd45α-mediated p38–NF-κB-mediated survival pathway (Gupta *et al.*, 2006). *Aggregatibacter actinomycetemcomitans* CDTB is known for its DNase activity and cell cycle arrest (Shenker *et al.*, 2007), which is compatible with the upregulation of *gadd45α* involved in DNA repair. The lack of an apoptotic phenotype, as shown in the annexin assay, suggest that interaction with *A. actinomycetemcomitans* whole cells may result in DNA damage, which may be repaired, leading to cell survival. Furthermore, infected cells exhibited higher levels (5.92 times) of transcripts of the anti-apoptotic factor BIRC3 (baculoviral IAP repeat-containing 3), which inhibits apoptosis by binding to the TNF receptor-associated factors TRAF1 and TRAF2, probably by interfering with activation of ICE-like proteases (Garrison *et al.*, 2009). The fast turnover of epithelial cells in the non-keratinized epithelium of the gingival crevice is a key factor in controlling the number of bacterial adherent cells. Therefore, the inhibition of cell cycle progression and decreased cell proliferation promoted by *A. actinomycetemcomitans* would probably maintain the colonizing internalized or adherent bacteria for longer periods.

Transcription of CCL2, encoding the inflammatory chemokine monocyte chemoattractant protein-1 (CCL2/MCP-1) was upregulated (12 times) in *A. actinomycetemcomitans*-infected gingival epithelial cells. High levels of MCP-1 were shown in the crevicular fluid of patients with periodontitis but not in healthy controls (Pradepp *et al.*, 2009). In general, MCP-1 is expressed by monocytes/macrophages, but it can also be produced by other cells, including epithelial cells, induced by the pro-inflammatory mediators TNF-α and IL-1, as well as by endotoxin and internalized bacteria (Jia *et al.*, 2008). In addition to chemotactic activity for leukocytes, MCP-1 also plays a role in modulation of cell proliferation, apoptosis, angiogenesis and bone remodeling (Yadav *et al.*, 2010). Recent study revealed that *P. gingivalis* was able to induce MCP-1 expression in human coronary artery endothelial cells induced by the transcription factor Erg-1 in concert with other transcription factors and cytokines (Maekawa *et al.*, 2010). On the other hand, *Streptococcus gordonii*, a commensal organism

frequently isolated from healthy subjects (Aas *et al.*, 2005), had no significant effect on the cytokine response, except for IL-8, and induced a minimal chemokine response in gingival epithelial cells, much lower than *P. gingivalis*, *A. actinomycetemcomitans* and *F. nucleatum*. Hence, commensals trigger a minimal inflammatory response (Stathopoulou *et al.*, 2010), while *A. actinomycetemcomitans* behaved as a true pathogen, eliciting a strong inflammatory response. The ELISA data in gingival tissues confirmed the *in vitro* data. However, subgingival *A. actinomycetemcomitans* levels were not correlated with levels of pro-inflammatory cytokines or ICAM-1 levels in gingival tissues. These data must be interpreted with caution because of the multiple bacteria colonizing the subgingival sites. Local inflammatory immune reactions of the host in response to periodontal pathogens seem to be decisive to protect the host against infection (Garlet *et al.*, 2003), but increased gene expression of inflammatory mediators persistent at an inflammatory site may result in pathological alterations in host tissues. In conclusion, our results showed that interaction between viable *A. actinomycetemcomitans* and epithelial cells results in transcription and expression of factors involved in immune cell recruitment and differentiation, including osteoclastogenesis, and these factors were shown in high levels in gingival tissues from periodontitis patients.

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