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Cytokine responses of human gingival fibroblasts to *Actinobacillus actinomycetemcomitans* cytolethal distending toxin

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Abstract

Actinobacillus actinomycetemcomitans is implicated in the pathogenesis of localized aggressive periodontitis, and has the capacity to express a cytolethal distending toxin (Cdt). Gingival fibroblasts (GF) are resident cells of the periodontium, which can express several osteolytic cytokines. The aims of this study were a) to investigate the role of Cdt in *A. actinomycetemcomitans*-induced expression of osteolytic cytokines and their cognate receptors in GF and b) to determine if the previously demonstrated induction of receptor activator of NFκB ligand (RANKL) by *A. actinomycetemcomitans* is mediated by these pro-inflammatory cytokines or by prostaglandin E_2 (PGE_2). *A. actinomycetemcomitans* clearly induced interleukin (IL)-6, IL-1β, and to a minimal extent, tumor necrosis factor (TNF)-α mRNA expression. At the protein level, IL-6 but not IL-1β or TNF-α expression was stimulated. The mRNA expression of the different receptor subtypes recognizing IL-6, IL-1β and TNF-α was not affected. A *cdt*-knockout strain of *A. actinomycetemcomitans* had similar effects on cytokine and cytokine receptor mRNA expression, compared to its parental wild-type strain. Purified Cdt stimulated IL-6, but not IL-1β or TNF-α protein biosynthesis. Antibodies neutralizing IL-6, IL-1 or TNF-α, and the PGE_2 synthesis inhibitor indomethacin, did not affect *A. actinomycetemcomitans*-induced RANKL expression. In conclusion, a) *A. actinomycetemcomitans* induces IL-6 production in GF by a mechanism largely independent of its Cdt and b) *A. actinomycetemcomitans*-induced RANKL expression in GF occurs independently of IL-1, IL-6, TNF-α, or PGE_2.

Keywords: *Actinobacillus actinomycetemcomitans*; Cytokines; Cytolethal distending toxin; Gingival fibroblasts

1. Introduction

Periodontitis is an inflammatory disease of infectious aetiology that leads to the destruction of tooth supporting tissues. The Gram-negative facultative anaerobe *A. actinomycetemcomitans* is associated with localized aggressive periodontitis, a form of periodontal disease occurring in young individuals and characterized by the rapid and severe loss of alveolar bone and connective tissue attachment. Bone loss is the end point of cellular mechanisms triggered to form and activate osteoclasts, the bone resorbing cells. Osteoclasts formation in inflammatory processes, such as marginal or apical periodontitis and rheumatoid arthritis, is suggested to occur via the enhanced expression of

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osteoclast-stimulating pro-inflammatory cytokines [1,2], including interleukin (IL) -1, IL-6 and tumor necrosis factor (TNF)-α, as well as prostanoids, including prostaglandin E_2 (PGE_2). Resident cells of the gingival connective tissue, namely the gingival fibroblasts (GF), together with infiltrating leukocytes, are considered a major source of these cytokines in periodontal disease [3]. Various *A. actinomycetemcomitans* components have been shown to stimulate the expression of cytokines and prostanoids by GF [4–14].

Interleukin-1, IL-6, TNF-α and PGE_2 are all known to regulate the expression of receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) in stromal cells and osteoblasts [15]. RANKL, a member of the TNF ligand superfamily, is expressed as a surface-associated ligand on osteoblasts in periosteal tissues, and stromal cells in haematopoetic tissues [16], as well as on T-lymphocytes [2,17]. It binds to its cognate receptor RANK on osteoclast progenitor cells [18], leading to their differentiation into multinucleated bone-resorbing osteoclasts [19,20]. This cellular interaction can be inhibited by OPG, a decoy receptor with homology to RANK, which is released by stromal cells and osteoblasts [21]. Previous reports have demonstrated a direct, or cytokine-mediated induction of RANKL in various host cells by lipopolysaccharide (LPS) from *Treponema denticola*, *A. actinomycetemcomitans* and *Escherichia coli* [22–25], by the gingipains of *Porphyromonas gingivalis* [26] and by the cytolethal distending toxin (Cdt) of *A. actinomycetemcomitans* [27].

The Cdt is the most recently described protein exotoxin of *A. actinomycetemcomitans* [28–30]. The Cdt family is known to induce growth arrest in various mammalian cells, by inflicting DNA strand-breaks and activation of DNA-damage checkpoint responses [31–33]. In terms of cytokine production, *A. actinomycetemcomitans* Cdt stimulates the synthesis of IL-1β, IL-6 and IL-8, but not TNF-α, macrophage colony-stimulating factor (M-CSF), or IL-12, by human peripheral blood mononuclear cells [34]. In a recent study, we demonstrated that *A. actinomycetemcomitans* induces RANKL expression by human GF, an event attributed to its Cdt [27]. However, the involvement of Cdt in *A. actinomycetemcomitans*-induced expression of pro-inflammatory cytokines, or their cognate receptors in GF has not been investigated. Moreover, it is not known whether the *A. actinomycetemcomitans*-induced RANKL expression in GF is mediated by inflammatory cytokines known to stimulate RANKL. Therefore, the aims of this study were a) to investigate the role of Cdt in the *A. actinomycetemcomitans*-regulated expression of pro-inflammatory cytokines and their receptors (IL-1R I, IL-1R II, TNF-R I, TNF-R II, IL-6R) in GF, and b) to determine if the *A. actinomycetemcomitans*-induced RANKL expression in GF is mediated by IL-1, IL-6, TNF-α, or PGE2.

2. Results

Analyses using semi-quantitative RT-PCR showed that the D7SS wild-type strain enhanced mRNA expression of IL-6, IL-1β, and to a lesser extent of TNF-α (Fig. 1). Extract from the D7SS *cdt*-mutant strain induced mRNA expression of these cytokines to an extent similar to the wild-type extract. None of the two bacterial extracts affected M-CSF mRNA expression.

The expression of the different receptor subtypes recognizing TNF-α, IL-1 and IL-6 was also investigated. As appears in Fig. 2, no differences could be observed in the mRNA expression of TNF-R I, TNF-R II, IL-1R I, or IL-6 R between the unchallenged controls and the cells challenged with either the D7SS wild-type strain, or the D7SS *cdt*-mutant strain. IL-1R II mRNA was not expressed by GF in any of the groups (data not shown).

To investigate if the enhanced mRNA expression of IL-6, IL-1β and TNF-α induced by *A. actinomycetemcomitans* resulted in increased protein expression, the amount of cytokines produced by the cells was analysed by ELISA. Secreted IL-1β and TNF-α were not detectable in any of the groups, after 24 h of bacterial challenge, whereas IL-6 protein secreted from the cells to the culture media was detected in the unchallenged GF culture supernatants and was significantly up-regulated in all bacterially challenged cultures (Table 1). The D7SS wild-type and *cdt*-mutant strains induced a 10-fold and 8.5-fold up-regulation of IL-6 protein,

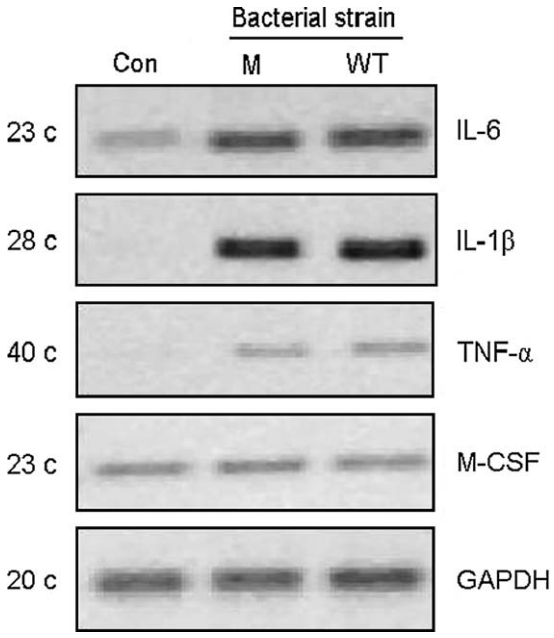

Fig. 1. Semi-quantitative RT-PCR analysis of IL-6, TNF-α, IL-1β and M-CSF mRNA expression in GF challenged with 1% *A. actinomycetemcomitans* extract. GF were cultured in the absence (Con) or presence of D7SS wild-type (WT), or D7SS *cdt*-mutant (M) extract, for 24 h. Number of PCR cycles is shown.