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Short communication

Soluble CD14 induces cytokine release by human oral epithelial cells

K. Feghali, S. Tanabe, D. Grenier
Groupe de Recherche en Écologie Buccale, Faculté de Médecine Dentaire, Université Laval, Quebec City, QC, Canada

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Background and Objective: The epithelial cell barrier is the first line of host defense against bacterial aggression in periodontal sites. In view of the fact that oral epithelial cells do not express membrane CD14 and that high levels of the soluble form of the CD14 receptor have been detected in the gingival crevicular fluid of patients with periodontitis, we investigated the effects of recombinant soluble CD14 (rsCD14), alone and in combination with *Aggregatibacter actinomycetemcomitans* lipopolysaccharide (LPS) on the inflammatory response of human oral epithelial cells.

Material and Methods: The oral epithelial cell line GMSM-K was stimulated with rsCD14, alone or in combination with *A. actinomycetemcomitans* LPS, and the levels of the inflammatory mediators interleukin (IL)-6, IL-8 and chemokine (C-C motif) ligand 5 (CCL5) were determined using ELISAs. Activation of the transcription factors nuclear factor-κB (NF-κB) and activator protein-1 was also monitored using ELISAs.

Results: rsCD14 significantly induced the secretion of IL-6, IL-8 and CCL5 by oral epithelial cells. The combination of rsCD14 and *A. actinomycetemcomitans* LPS augmented this effect. Activation of the NF-κB pathway was significantly increased in epithelial cells treated with rsCD14 compared with a nonstimulated control, whereas there was no effect on the activation of activator protein-1.

Conclusion: rsCD14 stimulated the inflammation cascade in oral epithelial cells, both alone or when associated with bacterial LPS, through an NF-κB-dependent pathway. This suggests that the presence of soluble CD14 in periodontitis lesions may contribute to the inflammatory process of periodontal disease.

Dr. Daniel Grenier, Groupe de Recherche en Écologie Buccale, Faculté de Médecine Dentaire, Université Laval, 2420 Rue de la Terrasse, Quebec City, QC, Canada G1V 0A6
Tel: (418) 656-7341
Fax: (418) 656-2861
e-mail: Daniel.Grenier@greb.ulaval.ca

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Introduction

Periodontal tissues face constant challenges from hundreds of commensal and pathogenic species of bacteria in the saliva and dental plaque. When these challenges exceed the capacity of the host to defend itself, the ensuing inflammatory and immune reactions lead to periodontitis, resulting in the destruction of tooth-supporting tissues and ultimately in tooth loss. Specific gram-negative bacteria, called periodontopathogens, have been associated with periodontal infections. The most frequently detected species in chronic periodontitis are *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia*, while *Aggregatibacter actinomycetemcomitans* has been associated with the aggressive forms of periodontitis (1). Lipopolysaccharides (LPS) are major cell-surface components of these bacteria, and high serum concentrations of LPS have been detected in patients with periodontitis (2). The presence of periodontopathogens and their products, such as LPS, in subgingival sites can stimulate both immune and mucosal

cells to produce cytokines and other proinflammatory mediators, which are at the origin of tissue destruction (3).

Cluster of differentiation antigen 14 (CD14) is a 55-kDa glycoprotein expressed on the surface of mature monocytes, macrophages and activated neutrophils (4). It is anchored to the plasma membrane by a phosphatidylinositol tail and lacks a transmembrane domain, making its role in cell signaling debatable (5). Membrane CD14 (mCD14) is a high-affinity receptor for bacterial LPS. For the interaction with mCD14 to take place, LPS binds to an opsonin (known as LPS-binding protein) that is produced by the liver, and the complex thus formed binds to mCD14 (6). CD14 is unable to initiate cell-signaling responses on its own, but it associates with toll-like receptor-4 and MD-2 to form a multiprotein LPS signaling receptor (7). Immune cells secrete inflammatory mediators, such as tumor necrosis factor-α, interleukin (IL)-1β, IL-6 and IL-8, in response to low concentrations of LPS, via nuclear factor-κB (NF-κB)-dependent and -independent pathways of the LPS-signaling cascade. Although activation of the LPS-signaling pathway plays an important role in host immunity against periodontal pathogens, excessive amounts of LPS signaling may lead to detrimental inflammatory responses followed by periodontal destruction (8).

The soluble form of CD14 (sCD14) has been detected in urine, serum, saliva and gingival crevicular fluid (9–13). sCD14 is slightly smaller than mCD14 and is believed to be the result of enzymatic shedding/cleavage of the membrane form of the receptor CD14 by specific proteases or phospholipases (14). Interestingly, elevated levels of LPS and sCD14 have been detected in the sera of patients with periodontitis, whereas the levels of sCD14 have been shown to decrease when periodontal inflammation is treated (15,16). A previous study in our laboratory showed that the outer membrane vesicles of *P. gingivalis* contribute to the loss of membrane-anchored CD14 receptors from macrophages. Furthermore, a comparative analysis of gingival crevicular fluid samples obtained from patients with different periodontal conditions revealed the presence of higher amounts of sCD14 in the periodontitis group than in healthy subjects (11). Earlier studies showed that sCD14 has the ability to interact with LPS, and that sCD14–LPS complexes can activate human cells that normally lack mCD14, such as epithelial cells, thus triggering an inflammatory response (17,18). In the present study, we hypothesized that sCD14, in the absence or presence of LPS, may help to amplify the inflammatory response during periodontitis by stimulating oral epithelial cells to secrete inflammatory cytokines. We also investigated the signaling pathways activated in epithelial cells in response to sCD14.

Material and methods

LPS preparation

A. actinomycetemcomitans ATCC 29522 was grown in Todd–Hewitt broth supplemented with 1% yeast extract. The cultures were incubated at 37°C in an anaerobic chamber (80% N_2, 10% H_2, 10% CO_2) for 24 h. LPS was isolated using the protocol described by Darveau & Hancock (19), which is based on the digestion of a whole-cell extract by proteinase K, followed by solubilization and precipitation steps. The LPS preparation was freeze-dried and stored at −20°C until used. The amount of contaminating protein comprised < 0.001% (w/w) of the LPS preparation.

Epithelial cell cultures and treatments

The immortalized human epithelial cell line, GMSM-K, developed by Valerie Murrah (Department of Diagnostics Sciences and General Dentistry, University of North Carolina at Chapel Hill, NC, USA), was used. Briefly, oral epithelial cells from a stillborn male fetus, of 30 wks' gestation, were grown in serum-free medium and transfected with the shuttle vector plasmid, pZ189, containing the T-antigen-coding region and replication origin from the Simian virus 40 (SV40) (20). This cell line has an epithelial phenotype, which was verified by electron microscopic and immunohistochemical analyses (20). The oral epithelial cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 μg/mL of penicillin–streptomycin), harvested by gentle trypsinization (0.05% trypsin–EDTA), washed once in DMEM supplemented with 1% FBS and suspended at a density of 4×10^5 cells/mL in DMEM supplemented with 1% heat-inactivated FBS. The cells were seeded in 12-well plates (4×10^5 cells/well in a total volume of 1 mL) and cultured overnight at 37°C in a 5% CO_2 atmosphere to allow cell adhesion before stimulation with recombinant soluble CD14 (rsCD14) (Feldan Bio Canada Inc., Montreal, QC, Canada) at concentrations ranging from 0 to 0.5 μg/mL, with or without the addition of 0.02 μg/mL of *A. actinomycetemcomitans* LPS. Stimulation with rsCD14 was also carried out in the presence of 10 μg/mL of polymyxin B. The doses used of rsCD14 and LPS were selected on the basis of preliminary experiments showing that LPS induced cytokine secretion by epithelial cells without affecting their viability, as determined using an MTT [3-(4,5-diethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (data not shown). After 24 h of incubation at 37°C in 5% CO_2, cell-free supernatants were collected and stored at −20°C until used.

Cytokine determination

Based on preliminary results related to cytokine secretion by the GMSM-K cell line, we investigated IL-6, IL-8 and chemokine (C-C motif) ligand 5 (CCL5) secretion following stimulation. Commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to quantify the concentrations of IL-6, IL-8 and CCL5 in the cell-free supernatants, according to the manufacturer's protocol. The absorbance at 450 nm was read using a microplate reader with the wavelength correction set at 550 nm. The rated sensitivities of the commercial ELISA kits were 9.3 pg/mL for IL-6, 31.2 pg/

mL for IL-8 and 15.6 pg/mL for CCL5.

Analysis of NF-κB p65 and activator protein-1 activation

To gain some insight into the mechanism of action of rsCD14, its effect on the activation of NF-κB p65 and activator protein-1 (AP-1) was investigated. These two pathways have been associated with the inflammatory response of human cells (21). Epithelial cells, prepared as described above, were incubated in the absence or presence of rsCD14 (0.02 and 0.1 μg/mL) for 1 h. Whole-cell extracts were then prepared using Nuclear Extract kits (Active Motif, Carlsbad, CA, USA), according to the manufacturer's protocol. They were adjusted to a protein concentration of 1 mg/mL using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA), which is similar to the well-documented Lowry assay. NF-κB p65 and AP-1 activation were determined in the cell extracts using TransAm™ NF-κB p65 and AP-1 kits (Active Motif). The 96-well plate of the ELISA-based kit contains immobilized oligonucleotides with an NF-κB p65 or an AP-1 consensus-binding site. Activated NF-κB p65 and AP-1 in the cell extract specifically bind to these oligonucleotides and were detected using specific antibodies.

Statistical analysis

Data are expressed as the means ± SD of three assays. The statistical analysis was conducted using the Student's *t*-test. A p-value of < 0.05 was considered statistically significant.

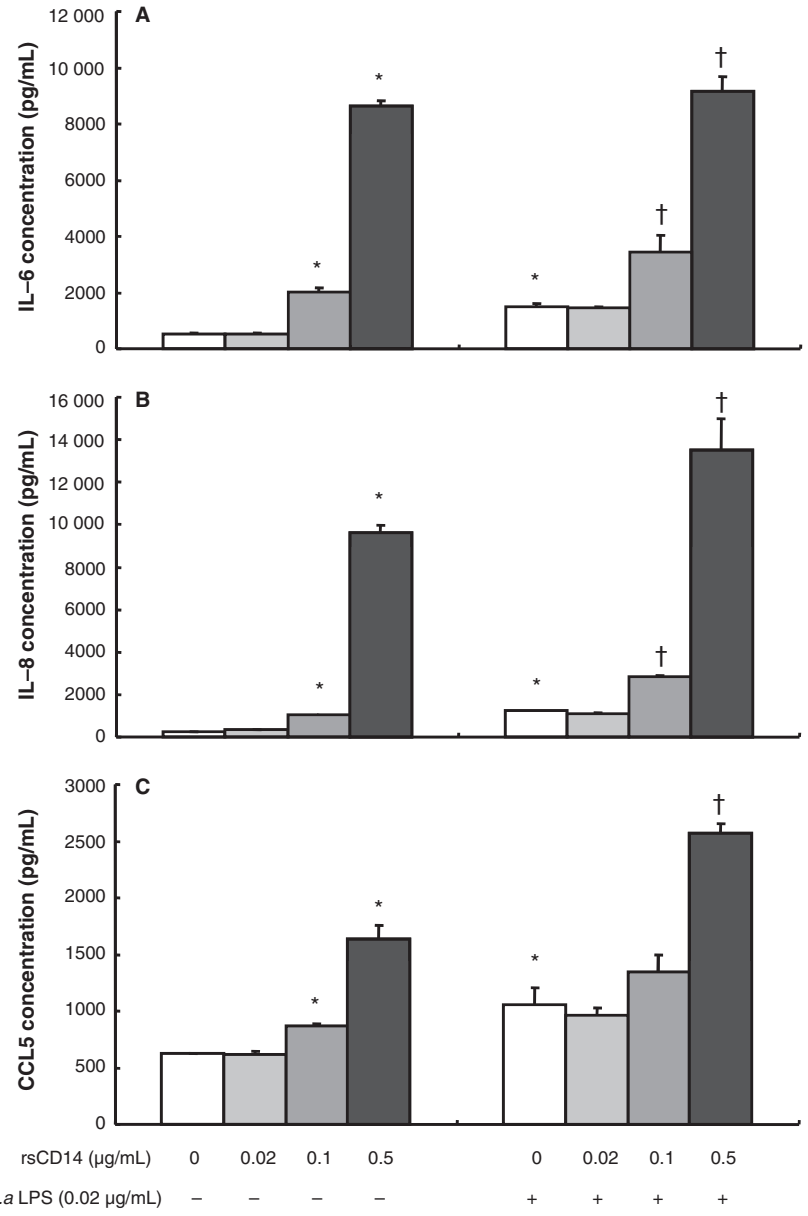

Fig. 1. Secretion of interleukin (IL)-6 (A), IL-8 (B) and chemokine (C-C motif) ligand 5 (CCL5) (C) by oral epithelial cells stimulated with recombinant soluble CD14 (rsCD14) in the absence and presence of *Aggregatibacter actinomycetemcomitans* (*A.a*) lipopolysaccharide (LPS). Data represent the mean ± SD of triplicate assays. *, significant increase compared with unstimulated control cells ($p < 0.05$); †, significant increase compared with LPS-stimulated cells ($p < 0.05$).

Results and Discussion

The effect of rsCD14 on cytokine secretion by oral epithelial cells is reported in Fig. 1. At the lowest concentration tested (0.02 μg/mL), rsCD14 did not trigger any significant secretion of IL-6, IL-8, or CCL5 by oral epithelial cells. However, stimulating cells with 0.1 and 0.5 μg/mL of rsCD14 resulted in a significant increase in the secretion of all three cytokines compared with unstimulated cells. More specifically, 0.5 μg/mL of rsCD14 increased the secretion of IL-6, IL-8 and CCL5 by 16-, 42- and 2.6-fold, respectively. Adding polymyxin B during the stimulation of epithelial cells with rsCD14 had no effect on cytokine secretion, indicating that the effect observed was not related to LPS contaminants (data not shown).

A possible synergistic effect of rsCD14 and *A. actinomycetemcomitans* LPS on the secretion of cytokines by oral epithelial cells was then investigated. Treating the cells with 0.02 μg/mL of LPS alone increased the secretion of IL-6, IL-8 and CCL5 compared with untreated control cells (Fig. 1). Stimulating oral epithelial cells with both rsCD14 and LPS resulted in some

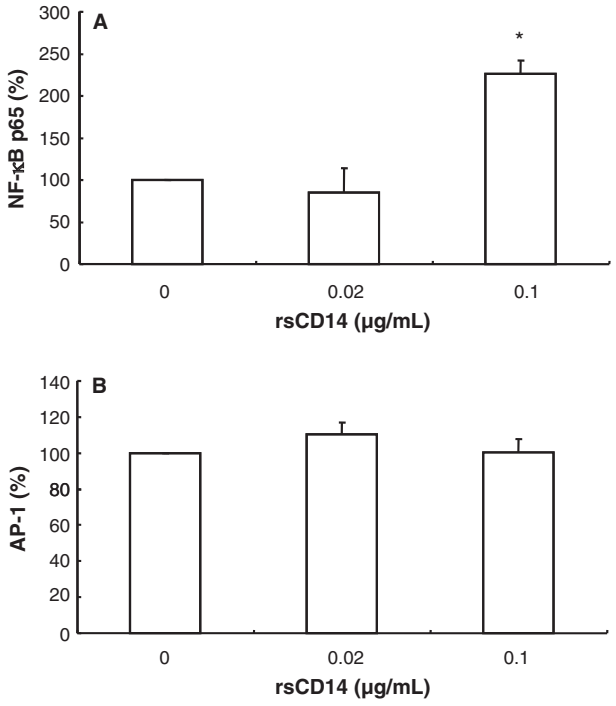

Fig. 2. Activation of the transcription factors nuclear factor-κB (NF-κB) (A) and activator protein-1 (AP-1) (B) by oral epithelial cells stimulated with recombinant soluble CD14 (rsCD14). Data represent the mean ± SD of triplicate assays. *, significant increase compared with unstimulated control cells ($p < 0.05$).

additive effects on cytokine secretion. However, no synergistic effect was observed.

The effect of rsCD14 on the activation of NF-κB p65 and AP-1 in oral epithelial cells was then tested in an attempt to understand, in greater detail, the mechanism of action of rsCD14. At 0.02 μg/mL, rsCD14 had no significant effect on either transcription factor (Fig. 2). However, the relative DNA-binding activity of NF-κB p65 in epithelial cells treated with 0.1 μg/mL of rsCD14 increased by up to 224 ± 21% compared with the unstimulated control (Fig. 2), while AP-1 was unaffected.

Controversial results have been reported on the responsiveness of epithelial cells following a challenge with LPS. While some epithelial cell lines are able to secrete IL-8 after being stimulated with LPS, others are unresponsive (22–24). The GMSM-K cell line used in our experiments showed a significant release of IL-6, IL-8 and CCL5 upon stimulation with LPS. As human oral epithelial cells do not normally express mCD14 (24), it is likely that the ability of LPS to elicit an inflammatory response in the GMSM-K cell line takes place through a CD14-independent mechanism. According to Striz *et al.* (25), adding a blocking agent known to be highly effective in inhibiting the binding of LPS to CD14 did not decrease the binding of LPS to epithelial cells.

GMSM-K cells were activated by the addition of rsCD14, alone and in the presence of *A. actinomycetemcomitans* LPS. Comparable results have been reported for human bronchial epithelial cells stimulated with *Escherichia coli* LPS, in that secretion of IL-6 and IL-8 was enhanced (25). However, our findings disagree with those of Uehara *et al.* (24), who reported that oral epithelial cells were not stimulated with bacterial components in the presence or absence of rsCD14 and serum. The specific receptors involved in the binding of sCD14 and its mechanism of action in oral epithelial cells are still unknown and warrant further investigation.

Interestingly, 0.1 μg/mL of rsCD14, in the absence of LPS, increased the activation of the NF-κB p65 heterodimer by greater than twofold compared with unstimulated cells. The inactive form of NF-κB p65 found in the cytoplasm of mammalian cells, which is usually activated by inflammatory inducers such as bacterial and viral components, is cleaved by proteases, and then translocates to the nucleus and binds to DNA (26). Activated NF-κB plays a role in the secretion of various inflammatory mediators and in the regulation of immunoreceptors.

There are many potential sources of sCD14 in periodontal pockets and saliva. Sugawara *et al.* (27) showed that human gingival fibroblasts secrete sCD14 after being stimulated with interferon-γ and bacterial LPS. Furthermore, sCD14 can also be released into the gingival crevicular fluid by the shedding/cleavage of mCD14 from CD14-expressing cells (11). Jin & Darveau (13) measured the levels of sCD14 in the gingival crevicular fluid of patients with variable degrees of untreated periodontitis and reported concentrations ranging from 0.16 to 51.74 μg/mL (mean concentration of 14.04 ± 4.15 μg/mL). Our data showed that oral epithelial cells secrete IL-6, IL-8 and CCL5 in response to as little as 0.1 μg/mL of rsCD14. These inflammatory mediators have been associated with the pathogenesis of periodontal disease through different mechanisms, including their ability to recruit neutrophils and other inflammatory cells to the inflammation site and their potential to enhance the secretion of matrix metalloproteinases, leading to bone resorption and destruction of periodontal tissues (28,29). Our results suggested that gingival epithelial tissues may contribute to periodontal damage through enhancement of the inflammatory response via a CD14-dependent mechanism *in vivo*.

While limited information is available on the exact contribution of sCD14 to, and its mechanism of action in, the pathogenesis of periodontal disease, it can be speculated that sCD14 plays a significant role because it is detected in elevated amounts in the gingival crevicular fluid of patients

with periodontitis (11). sCD14 interacts with epithelial cells that do not normally express endogenous CD14 (30,31). Our study showed that rsCD14 enhances the inflammatory response of oral epithelial cells through the secretion of inflammatory mediators. However, it has been proposed that sCD14 may also play a protective role by controlling gram-negative bacterial infections through its capacity to bind LPS (32) and compete with the membrane receptor CD14. For instance, sCD14 reduces the mortality of mice from endotoxin shock as well as the severity of intramammary *E. coli* infections (33). Studies investigating a possible protective role of sCD14 in periodontal disease are necessary.

As the epithelial barrier is the first line of defense against oral microflora, it is crucial to understand the behavior of epithelial cells and their responses to the continuous changes and challenges in their microenvironment. Periodontitis has been associated with elevated levels of sCD14 in the sera and gingival crevicular fluid of patients (11,15,16). According to our data, sCD14 may activate human oral epithelial cells through the NF-κB p65 pathway, leading to increased secretion of the inflammatory mediators IL-6, IL-8 and CCL5. The addition of LPS from a major periodontopathogen (*A. actinomycetemcomitans*) enhanced the inflammatory responses of these cells. Our study suggests that the role of sCD14 in periodontal inflammation lesions is significant and cannot be overlooked. Additional *in vitro* and *in vivo* studies are required to gain a better understanding of the role of sCD14 in periodontal disease.

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