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Cysteine proteases from *Porphyromonas gingivalis* and TLR ligands synergistically induce the synthesis of the cytokine IL-8 in human artery endothelial cells

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ABSTRACT

Objective: Bacterial pathogens are frequently detected in atheromatous lesions, however, their contribution to atherosclerosis remains unknown. The present study was aimed to explore the effect of the *P. gingivalis* cysteine protease gingipain towards the proinflammatory response of human aortic endothelial cells (HAECs).
Design: HAECs were exposed to gingipains (*Rgps*) extracted from the oral pathogen *P. gingivalis*. In addition, HAECs were co-stimulated with the TLR ligands *P. gingivalis* LPS, *E. coli* LPS or heat-killed *P. gingivalis* (HKPG) in combination with gingipain-active or gingipain-inactive extracts. After stimulation, IL-8 mRNA expression and protein synthesis were analysed by RT-PCR and ELISA. Means and standard errors were computed following by statistical testing ($P \leq 0.05$).
Results: In HAECs, *Rgps* significantly increased the IL-8 mRNA (5.8 ± 1.1-fold) and protein expression (523.0 ± 57.5 pg/ml) compared to untreated controls. Co-stimulation experiments showed a significant synergistic effect for the IL-8 mRNA expression and protein synthesis when HAECs were exposed to a combination of the purified TLR ligands (*P. gingivalis* or *E. coli* LPS) or HKPG and gingipain-active extracts.
Conclusions: These results demonstrated the synergistic effects of TLR ligands and *P. gingvalis* cysteine proteases for the proinflammatory responses in artery vascular endothelial cells and highlight a mechanism by which bacteria may contribute to the development of atherosclerosis.

1. Introduction

Inflammatory processes have become an integral part of the pathophysiology of atherosclerosis and are presumed to be involved in all stages of atherosclerosis from the initiation, progression to the final stages of infarction.[1] Chronic infections are thought to be involved in the pathogenesis of cardiovascular diseases by releasing cytokines and pro-inflammatory mediators (e.g. C-reactive protein) that may initiate a cascade of inflammatory reactions causing

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endothelial damage and cholesterol plaque formation.[2] Healthy endothelium does not allow the attachment of free leukocytes, however, initial damage of endothelial cells induces the expression of vascular cell and intercellular adhesion molecules followed by the aggregation of leukocytes to the endothelium.[3,4] The expression of adhesion molecules in human artery endothelial cells (HAEC) and the subsequent aggregation of leukocytes induce the release of chemokines and chemoattractants in vascular smooth muscle cells.[5] This cascade leads to the recruitment of monocytes and T-cells into the arterial wall at sites with deposits of low-density lipoproteins. Thus, pro-inflammatory cytokines produced by HAECs are related to atherosclerosis and in particular interleukin-8 (IL-8) plays a major role in the adhesion of monocytes to human atherosclerotic lesions.[6]

Periodontitis is a chronic inflammatory disease that affects gingival and periodontal tissues leading to the loss of periodontal attachment and alveolar bone. It has been shown that untreated periodontitis is the most causative reason for tooth loss.[7] Although, periodontal diseases do not show characteristics of a specific infection, some oral pathogens, such as *Porphyromonas gingivalis*, *Bacteroides forsythus*, and *Aggregatibacter actinomycetemcomitans*, are commonly detected in aggressive forms of periodontitis, and those bacterial species have been proposed as major etiological factors in the pathogenesis of chronic periodontitis.[8] Several epidemiological studies have provided evidence that oral infections, such as periodontitis, may be an independent risk factor for systemic diseases, such as coronary heart disease, which is the most important clinical manifestation of atherosclerosis and the leading cause of death worldwide in the last century.[9] Periopathogens like *P. gingivalis* have been frequently detected in atherosclerotic plaques indicating a potential causal relationship between oral inflammatory processes and vascular diseases.[10–13]

P. gingivalis is an anaerobic Gram-negative bacterium that has been isolated from periodontal pockets. It produces a variety of virulence factors, such as lipopolysaccharides (LPS), fimbriae, toxic products of metabolism and proteases, which stimulate host cells to release inflammatory mediators.[14] Gingipains are cysteine proteases derived from *P. gingivalis* that have been recognized as major virulence factors for the development of periodontal disease. Two kinds of cysteine proteases were identified: the arginine-specific protease referred to as arg-gingipain (*Rgp*; subtypes *RgpA* and *RgpB* with a molecular weight of 50 and 95 kDa, respectively) that cleaves peptide bonds specifically at arginine residues as well as the lysine-specific protease (lys-gingipain, *Kgp*) with a molecular weight of 105 kDa that cleaves peptide bonds specifically at lysine residues.[15] Gingipains have shown to synergistically induce the synthesis of pro-inflammatory mediators by activation of protease-activated receptors (PAR) in combination with Toll-like receptors (TLR) in human monocytes. This mechanism may also be relevant in vascular pathology by linking the presence of bacteria with the formation of atheromatous plaques.[16] However, the mechanisms by which bacterial virulence factors participate in the initiation and propagation of inflammatory processes relevant in atherosclerosis are unclear. The aim of the present study was to explore the role of the cysteine protease gingipain synthesized by the oral pathogen *P. gingivalis* during proinflammatory responses of HAECs. Therefore, it was of interest to elucidate potential synergistic effects of gingipains for the release of the proinflammatory mediator IL-8 in TLR-activated HAECs.

2. Materials and methods

2.1. Materials

Purified LPS from *P. gingivalis* ATCC 33277 (10^4 EU/mg; Endotoxin Units) and heat-killed whole cells of *P. gingivalis* ATCC 33277 (125 EU/ml) were purchased from InvivoGen (San Diego, CA, USA). L-Cysteine, 100% ethanol and DMSO were obtained from Merck (Darmstadt, Germany). N-Benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA), LPS from *E. coli* serotype 026:B6 (5×10^5 EU/ml), leupeptin, Z-VAD-FMK, 2-mercaptoethanol and ethidium bromide were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Preparation of gingipain extracts from P. gingivalis

Gingipain active extracts were prepared according to previously published protocols.[17] Briefly, *P. gingivalis* strain ATCC 33277 was grown in Brucella-Bouillon and Brucella-Agar supplemented with hemin (5 μg/ml), vitamin K (0.5 μg/ml), and L-cysteine (0.1%). Bacterial cultures were incubated at 37 °C under anaerobic conditions (10% H_2, 10% CO_2 and 80% N_2). Photometric measurements were performed to monitor optical densities (OD; late logarithmic growth phase) of *P. gingivalis*. After an OD of 1.0 was reached, bacteria were centrifuged at 12,000 × *g* for 45 min at 4 °C and filtered through a 0.45-μm-pore filter (Millipore, Bellerica, USA). The extracellular culture fluid was precipitated at −20 °C in a 60:40 ratio of acetone to cell-free medium, with constant stirring over a period of 15 min. The temperature of the solution was maintained below 0 °C using an ice bath. The precipitate was centrifuged at 12,000 × *g* for 30 min at 4 °C, and the pellet was resuspended in a solution containing 150 mM NaCl, 20 mM Bis–Tris and 5 mM $CaCl_2$. The resuspended pellet was dialyzed overnight at 4 °C in a Spectrapor 12,000- to 14,000-molecular-weight cut-off dialysis tubing versus 4 l of the same buffer containing Aldrithiol-4 for stabilization of the gingipain extract. This procedure was followed by three additional changes of dialysis buffer without Aldrithiol-4. After dialysis, the sample was centrifuged at 34,000 × *g* for 1 h at 4 °C and the resulting supernatant was concentrated in a pressurized stirring concentrator (Millipore) with a 10,000-molecular-weight-cut-off membrane at 4 °C. The concentrated gingipain extract was clarified by centrifugation at 192,000 × *g* for 1 h at 4 °C and stored in aliquots at −80 °C.

2.3. Gingipain concentration and protease assay

The concentration of the gingipain extract was determined by the Pierce Protein Assay (Rockford, IL, USA) following the protocol of the manufacturer.

To determine gingipain activity (Rgp and Kgp), 5 μl of the gingipain extract was pre-incubated in a final volume of 150 μl assay buffer. The reaction was initiated by the addition of 50 μl of 4 mM BAPNA for Rgp activity, or acetyl-lysine-*p*-nitroanillide, for Kgp activity. The rate of enzymatic substrate hydrolysis was