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Interferon-γ stimulates CD14, TLR2 and TLR4 mRNA expression in gingival fibroblasts increasing responsiveness to bacterial challenge

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ABSTRACT

Objective: To investigate the potential effects of IFN-03A5 on the responsiveness of human gingival fibroblasts to bacterial challenge.
Design: mRNA and protein expression of CD14, TLR2 and TLR4 in human gingival fibroblasts was detected by quantitative polymerase chain reaction (Q-PCR) and flow cytometry. The effect of preincubation with IFN-03A5 on subsequent bacterial LPS-induced expression of IL-6 and IL-8 by gingival fibroblasts was determined by ELISA. Bacterial LPS-induced IκBα degradation in human gingival fibroblasts was investigated by western blot.
Results: Human gingival fibroblasts express CD14, TLR2 and TLR4 mRNAs. IFN-03A5, but not IL-103B2, induced mRNA expression of all three receptors and the expression of membrane bound CD14 protein. Pre-incubation of fibroblasts with IFN-03A5 and subsequent stimulation with *Escherichia coli* LPS or *Porphyromonas gingivalis* LPS led to increased production of IL-6 and IL-8. LPS-induced pro-inflammatory cytokine production was abrogated by a blocking antibody to CD14. Both *E. coli* LPS and *P. gingivalis* LPS induced IκBα degradation in human gingival fibroblasts.
Conclusion: Our data indicate that IFN-03A5 primes human gingival fibroblasts, through the upregulation of CD14 expression, which results in increased responsiveness to bacterial LPS challenge, as determined by pro-inflammatory cytokine production.

1. Introduction

Periodontal disease is a chronic inflammatory condition resulting from complex interactions between periodontal pathogens and the host tissues, leading to the loss of the tooth-supporting structures. These primarily Gram-negative pathogens possess an array of structural or secreted components which stimulate host cells to activate a range of inflammatory responses (Giannobile, 2008). These components are termed pathogen-associated molecular patterns (PAMPs) and include lipopolysaccharide (LPS), lipoproteins, and peptidoglycans on the bacterial cell surface. *Porphyrmonas gingivalis*, and specifically its LPS, is thought to play a key role in the pathogenesis of periodontal disease (Herath et al., 2013). In mammals, as part of the innate immune response, PAMPs are recognised by host cells through pattern recognition molecules including the Toll-like receptors (TLRs), a family of membrane-bound and intracellular receptors (Browne, 2012). Variations in LPS structure among bacteria impact on the bioactivity of LPS molecules, with the number of lipid A acyl chains and their carbon length affecting LPS activity (Stover et al., 2004). The chemical structure of *Escherichia coli* lipid A is considered to be the most optimally recognised among bacterial lipid A structures (Rietschel et al., 1994). *E coli* LPS induces a biological response by interacting with specific cell surface receptors such as cluster of differentiation 14 (CD14) and TLR4. CD14 is thought to play a central role, acting as a receptor for LPS bound to lipopolysaccharide binding protein (LBP). However, CD14 lacks transmembrane and intracellular domains, and is therefore unable to transport signals through the cell membrane. Rather, CD14 functions to present LPS to members of the TLR family, inducing intracellular signalling (Pfeiffer et al., 2001; Schmitz & Orso, 2002). Of specific interest to periodontal inflammation are TLR2 and TLR4 because *P. gingivalis* LPS has previously been reported to signal *via* both receptors (Darveau et al., 2004). However a recent publication indicates that TLR2 activation is due at least in part to lipoprotein lipase sensitive co-purifying molecules. In this study TLR2 activation was independent of lipid A structures and intact

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P. gingivalis bacterial cells treated with lipoprotein lipase were unable to activate TLR2 (Jain, Coats, Chang, & Darveau, 2013).

The nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signalling pathway is a major intracellular pathway activated by environmental stimuli such as periopathogenic LPS. In non-stimulated cells, NFκB remains inactive in the cytoplasm, bound to inhibitor of kappa B (IκB). Following stimulation, IκB kinases (IKKα and IKKβ) are activated and subsequently phosphorylate the IκB proteins, leading to their degradation through a ubiquitin-proteasome-dependent mechanism. Proteolysis of IκB, leads to the release of NFκB, allowing translocation to the nucleus where it activates the transcription of numerous genes, including inflammatory cytokines (Bhoj & Chen, 2009).

Fibroblasts are the major cell type found in the periodontal tissues and are responsible for the synthesis and degradation of connective tissue, maintaining structural integrity. Gingival fibroblasts produce various proinflammatory cytokines, including IL-1β, IL-6 and IL-8, following interaction with bacterial ligands, including LPS, and are involved in the pathogenesis of chronic inflammatory periodontal disease (Lerner, 2005). These cells constitutively express CD14 and TLRs, although reports on the expression of specific TLRs, including TLR2, on gingival fibroblasts are contradictory (Hatakeyama et al., 2003; Putnins, Sanaie, Wu, & Firth, 2002). The expression of these receptors can also be regulated by inflammatory mediators. Treatment of oral epithelial cells with the inflammatory cytokine gamma interferon (IFN-γ) enhances their cytokine production in response to LPS activation, through upregulation of TLR2 and TLR4 expression (Uehara, Sugawara, & Takada, 2002). Initial studies on gingival fibroblasts suggest IFN-γ may also regulate CD14 and TLR4 expression (Mochizuki et al., 2004; Tamai et al., 2002). Thus, in the inflamed tissue environment, cytokine-induced expression of membrane bound receptors on gingival fibroblasts could lead to an increased responsiveness to LPS signalling. This study investigates the effects of IFN-γ on CD14, TLR2 and TLR4 mRNA expression by gingival fibroblasts and the subsequent responsiveness of gingival fibroblasts to *E. coli* and *P. gingivalis* LPS activation following interaction with IFN-γ.

2. Material and methods

2.1. Cell culture

Human gingival fibroblast populations were developed by explant culture from clinically healthy gingival tissue removed during crown lengthening procedures. Ethical approval for the study was granted by the Office of Research Ethics Committees of Northern Ireland. All patients were fully informed of the study and gave informed consent for the use of the redundant tissue.

Excised tissue was cut into pieces approximately 2 mm^3 in size and explanted in culture flasks (Sarstedt, Leicester, UK) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), L-glutamine (2 mM), and antibiotics (penicillin (100 U/ml), streptomycin (100 μg/ml) (Invitrogen, Paisley, UK). Culture flasks were incubated at 37 °C in a humidified atmosphere with 5% CO_2 and the culture medium was changed every three days. On reaching confluence, fibroblasts were harvested by trypsinisation and subcultured; cells were used between passages 4 and 7 for all studies. All studies were carried out on the same gingival fibroblast strain.

2.2. ELISA

Gingival fibroblasts were plated at a density of 8×10^4 cells into separate wells of 24-well tissue culture plates and grown in DMEM culture media supplemented with 10% FCS and 100 μg/ml penicillin/streptomycin for 48 h. The cells (at approximately 70% confluence) were washed twice in Hank's buffered salt solution and then stimulated by *E. coli* LPS (0.01–10 μg/ml) (catalogue number L2630, Sigma–Aldrich, Poole, UK) or *P. gingivalis* LPS (0.01–10 μg/ml) (catalogue code tlrl-pglps, Invivogen, San Diego, CA) for 24 h. Culture conditioned medium was collected and centrifuged at 2500 rpm, 900 g for 10 min. DuoSet ELISA Development kits (R&D systems, Abingdon, UK) were used for detection of IL-6 and IL-8 in the supernatants. In 'priming' studies, fibroblast cultures were incubated with IFN-γ (100 ng/ml) (catalogue number I3265, Sigma–Aldrich, Poole, UK) for 48 h, prior to addition of LPS. The 100 ng/ml concentration of IFN-γ was used on the basis of findings of preliminary experiments (data not shown).

Neutralizing antibodies – anti-human TLR4 antibody (catalogue code PAb-hTLR4, Invivogen, San Diego, CA), anti-human CD14 antibody (catalogue number M0825, DakoCytomation, Glostup, Denmark) or an isotype control antibody (catalogue number ab18456 Abcam plc. Cambridge, UK) – were used as per manufacturer instructions to determine the function of the individual receptors on LPS signalling post-IFN-γ activation, fibroblast cell cultures were incubated with the appropriate antibody for 1 h prior to LPS stimulation for 24 h.

2.3. Western blots

Fibroblasts were grown to confluence in 6-well plates, washed and incubated with *E. coli* LPS (100 ng/ml) and *P. gingivalis* LPS (10 μg/ml) over a 2 h study period. At specific timepoints, whole-cell lysates were obtained by washing fibroblast monolayers with ice-cold phosphate buffered saline (PBS). Fifty μl of lysis buffer (RIPA buffer from Sigma–Aldrich containing 10% protease inhibitor

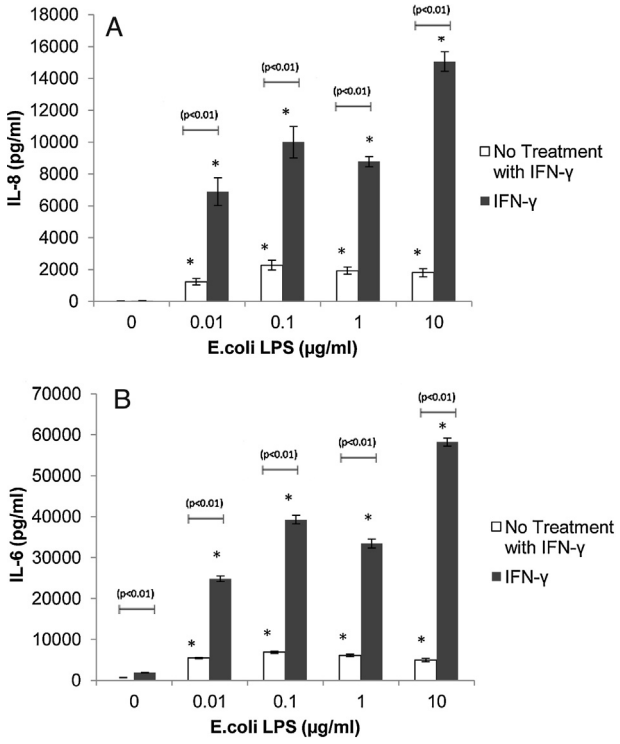

Fig. 1. *E. coli* induces IL-6 and IL-8 production in gingival fibroblasts. Gingival fibroblasts were incubated in medium, or medium supplemented with IFN-γ (100 ng/ml) for 48 h, prior to incubation with *E. coli* LPS over the concentration range 0–10 μg/ml for 24 h protein production for (A) IL-8 and (B) IL-6 were determined. The data represent mean ± SEM of triplicate determinations for each treatment group (* indicates a statistically significant difference $p < 0.01$ when compared to the relevant 0 μg/ml group).