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Porphyromonas gingivalis stimulates TACE production by T cells

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Introduction: Tumour necrosis factor-α converting enzyme (TACE), also known as ADAM17, is a membrane-bound metalloprotease and disintegrin. It is produced by a number of host cells and is known to shed and release cell-bound cytokines, particularly members of the tumour necrosis factor family. The aim of this study was to investigate the effect of *Porphyromonas gingivalis* on TACE production by a human T-cell line, to identify putative virulence factors involved in this process, and to investigate the effect of doxycycline.
Methods: *P. gingivalis* 6-day culture supernatants were used to challenge Jurkat T cells for 6 h. Secreted and cell-associated TACE levels were measured by enzyme-linked immunosorbent assay, whereas messenger RNA expression was investigated by quantitative real-time polymerase chain reaction. To investigate the involvement of cysteine proteases or proteinaceous components in general, *P. gingivalis* culture supernatants were treated with the specific chemical inhibitor TLCK or heat-inactivated, respectively. The effect of doxycycline on the regulation of TACE secretion by *P. gingivalis* was also investigated.
Results: *P. gingivalis* challenge resulted in a concentration-dependent enhancement of TACE messenger RNA expression and protein release by Jurkat cells. TLCK treatment or heat treatment of *P. gingivalis* culture supernatants decreased TACE release to control levels. Doxycycline inhibited TACE secretion dose dependently.
Conclusion: The induction of TACE by T cells in response to *P. gingivalis* may in turn favour the shedding of host cell-bound cytokines into the local microenvironment, potentially amplifying the inflammatory response. In the present experimental system, *P. gingivalis* cysteine proteases are involved in TACE release by T cells.

Key words: periodontitis; *Porphyromonas gingivalis*; T cells; TACE

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Introduction

Porphyromonas gingivalis is a gram-negative black-pigmented anaerobe, highly implicated in chronic periodontitis in humans (1). This species has also been shown to induce experimental periodontitis in animal models (2). The main putative virulence factors of this pathogen are its lipopolysaccharide (LPS) (3) and its Arg-X and Lys-X cysteine proteases (gingipains) (4), which are capable of perturbing local immunity and deregulating the host's inflammatory response (5, 6). These virulence properties of *P. gingivalis* may favour its capacity to invade the deeper periodontal tissues and establish chronic inflammation (7).

Tumour necrosis factor-α converting enzyme (TACE) is a member of the 'A Disintegrin And Metalloprotease' (ADAM) family, namely ADAM17, with a cysteine-rich, a disintegrin and a zinc-dependent metalloprotease domain. It is constitutively expressed by several cell types, including T cells and monocytes, either as a secreted, or a membrane-bound protein (8–11). Bacterial challenge is considered a major regulator of TACE expression (12, 13). As a global 'sheddase', TACE aims at the

proteolytic cleavage and release of the ectodomain of cell-membrane bound cytokines. In this manner, TACE can efficiently mobilize cytokines into the local microenvironment, which in turn can act in a paracrine manner to amplify their effects and establish inflammation (14). The substrates that TACE is able to shed are ligands and receptors highly associated with inflammation, such as tumour necrosis factor-α and its p55 receptor, transforming growth factor-α, interleukin-1 receptor II, receptor activator of nuclear factor-κB ligand, and epidermal growth factor receptor ligands (8, 11, 15, 16).

The involvement of TACE in rheumatoid arthritis (17) and tumour progression (18, 19) has been clearly demonstrated, and this enzyme is now considered a potential target for drug development (16, 20). It was recently demonstrated that TACE may be of relevance to periodontal disease (21). It is detected in human gingival crevicular fluid, and its levels are elevated in periodontitis, compared with patients with gingivitis or healthy subjects. Importantly, patients with periodontitis who are undergoing immunosuppressive treatment exhibit particularly low gingival crevicular fluid TACE levels, indicating that T cells are a major source of this enzyme. This is perhaps not surprising because T cells have a key role in the pathogenesis of periodontal disease (22, 23).

Inhibition of TACE is considered an effective pharmacological target in inflammatory and neoplastic diseases (16), and potentially in periodontal diseases (24). Doxycycline is an antibiotic that has also been shown to exert anti-inflammatory properties, distinct from its antimicrobial ones (25). These properties have been used clinically in the treatment of inflammatory diseases (26), including periodontitis (27). Recent work using an *in vivo* infection model suggests that doxycycline may attenuate inflammation partly by targeting TACE, resulting in reduced cytokine release (28).

Nevertheless, the mechanisms of TACE regulation in response to periodontal pathogens have not been investigated. *P. gingivalis* is a potent inducer of T-cell responses (29, 30) so there is merit to investigating TACE regulation in an *in vitro* system that involves *P. gingivalis* and T cells. Therefore, the aim of this study was to investigate if *P. gingivalis* regulates TACE in a T-cell line, and to evaluate the putative involvement of its virulence factors in this. Furthermore, the effect of sub-antimicrobial doses of doxycycline on TACE regulation by *P. gingivalis* was investigated.

Materials and methods

Cell cultures

Jurkat T-lymphocyte leukaemia cells (E6-1; American Type Tissue Culture Collection, Manassas, VA) were maintained in RPMI Glutamax (Gibco BRL Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum. The cells were harvested in the mid-log growth phase, and plated in 12-well tissue culture plates, at a density of 10^6 cells/well in 1 ml culture medium. For the experiments, the cells were cultured in the presence or absence of *P. gingivalis* culture supernatants for 6 h.

Bacterial cultures and growth conditions

P. gingivalis W50 wild-type strain was cultured in blood agar base supplemented with 5% horse blood (Oxoid, Hampshire, UK) and maintained by weekly subculture for up to 5 weeks. Liquid cultures were prepared by inoculation of bacterial colonies (3–4 days old) from blood agar plates into 10 ml Brain–Heart infusion broth (Oxoid) supplemented with 5 mg/l haemin (Sigma, Poole, UK), and incubated for 24 h. Ten per cent inoculum was transferred to 90 ml of the same medium and incubated for 6 days. All cultures were grown at 37°C in a Don Whitley anaerobic cabinet, MACS MG500, in an atmosphere of 80% N_2, 10% H_2 and 10% CO_2. After this culture period, bacteria were harvested by centrifugation at 10,000 ***g*** for 15 min at 4°C and supernatants were collected, filter-sterilized over a 0.2-μm filter, and stored at −80°C until use. These *P. gingivalis* preparations were diluted in the cell-culture medium, and their concentration is expressed as total bacterial protein (μg/ml) present in the cell cultures. Protein concentration was determined using a Bio-Rad Protein assay (Bio-Rad, Hemel Hempstead, UK).

Treatments of *P. gingivalis* culture supernatants and LPS preparation

To investigate the role of *P. gingivalis* cysteine proteinases in TACE production, the *P. gingivalis* W50 culture supernatant was pretreated with 1 mM of the proteinase inhibitor Na-*p*-tosyl-llysine chloromethyl ketone hydrochloride (TLCK) (Sigma-Aldrich, Poole, UK) for 1 h at 4°C, before addition to the Jurkat cell cultures. To investigate the role of proteinaceous components in general, *P. gingivalis* W50 culture supernatant was heat-treated at 70°C for 1 h, to inactivate the protein content, before challenging the cells. Purified *P. gingivalis* LPS was prepared as previously described (6).

Treatment of the cell cultures with doxycycline

The effect of doxycycline on the regulation of TACE secretion by the cells was also investigated. For this purpose, concentrations of doxycycline (Sigma-Aldrich) up to 10 μg/ml were administered to the cells concomitantly with *P. gingivalis*, for 6 h of challenge.

Cytotoxicity assay

The cytotoxic effects of *P. gingivalis* on Jurkat cells were evaluated by measuring the extracellularly released cytosolic lactate dehydrogenase, using the colorimetric CytoTox96® Non-Radioactive Cytotoxicity Assay, according to the manufacturer's instructions (Promega, Southampton, UK).

Extraction of total RNA and synthesis of complementary DNA

Total RNA was extracted from cells using RNeasy Mini kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. RNA was quantified using a Nanodrop spectrophotometer and reversed transcribed into complementary DNA. One microgram of total RNA was incubated with 0.5 μg/ml of oligo-dT primer (Promega) at 70°C for 5 min and cooled on ice. Master mix was added to samples, comprising 10 mM dNTPs, 200 units moloney murine leukaemia virus reverse transcriptase enzyme and buffer (Promega), and distilled H_2O to a final volume of 25 μl. For the reverse transcription reaction, these samples were incubated at 40°C for 60 min, 70°C for 15 min and then cooled down to 4°C.

Quantitative real-time polymerase chain reaction PCR

Quantification of TACE messenger RNA (mRNA) expression levels in the prepared complementary DNA samples was performed by quantitative real-time polymerase chain reaction (qPCR) TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA). The assay IDs were ADAM17/TACE: Hs01048105-m1 and 18S ribosomal RNA (rRNA): Hs99999901-s1. The 18S rRNA served as an endogenous RNA control. For the

amplification reactions a qPCR Master Mix was used (Abgene, Epsom, UK), and the qPCR analyses were performed in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The amplification conditions were 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The expression levels of TACE transcripts were calculated by using the comparative Ct method ($2^{-\Delta Ct}$ formula) after normalization to 18S rRNA.

Determination of TACE levels by enzyme-linked immunosorbent assay

After 6 h of challenge with *P. gingivalis* culture supernatants, the Jurkat T-cell cultures in suspension were centrifuged at 123 ***g*** for 5 min. The resulting supernatants were collected and TACE concentrations were evaluated by a human-specific TACE enzyme-linked immunosorbent assay (ELISA) kit, in accordance with the manufacturer's instructions (DuoSet ELISA Development kit, R&D Systems, Abingdon, UK). In a series of experiments, the cell-associated fraction was also analysed for TACE content. After centrifugation of the cell cultures in suspension, while the supernatant was collected for evaluation of secreted TACE, the cell pellets were washed with cold phosphate-buffered saline, and suspended in chilled lysis buffer (150 mM sodium dodecyl sulphate, 50 mM Tris–HCl pH 7.3, 2.5% sodium chloride, 1% nonidet P-40), including 1 : 100 Protease Inhibitor Cocktail (Sigma), for 20 min at 4°C. The resulting cell lysates were collected and centrifuged at 277 ***g*** for 20 min at 4°C to remove cell debris.

Statistical analysis

The significance of differences between control and test groups was assessed by one-way analysis of variance, and Bonferroni *post-hoc* test. *P*-values <0.05 were considered indicative of statistical significance. The data are expressed as means ± standard error of means (SEM).

Results

Jurkat T cells were challenged with ascending protein concentrations of *P. gingivalis*, and the effect on cell death was determined after 6 h, by measuring the extracellularly released lactate dehydrogenase. Bacterial concentrations equal to or lower than 6.4 μg/ml did not elicit any cytotoxicity, compared with control (data not shown).

The effect of *P. gingivalis* on TACE mRNA expression and protein secretion by the cells was then investigated after 6 h of challenge. Compared with the unchallenged control, *P. gingivalis* significantly enhanced TACE mRNA expression (Fig. 1) and protein secretion (Fig. 2) in a concentration-dependent manner. Indicatively, 6.4 μg/ml *P. gingivalis* caused a 2.3-fold upregulation compared with the control.

The next step was to identify *P. gingivalis* virulence factors potentially involved in the upregulation of TACE. For this purpose, *P. gingivalis* culture supernatants (6.4 μg/ml) were heat-inactivated or TLCK-treated before challenging the cells, to destroy the proteinaceous components, or inhibit the cysteine proteinase activity, respectively. After 6 h of challenge, both treatments resulted in abolishment of TACE secretion to control levels (Fig. 3). The TLCK treatment alone did not affect TACE secretion by the cells, compared to the control (data not shown). The effect of purified *P. gingivalis* LPS was also tested. Concentrations as high as 1000 ng/ml failed to enhance TACE secretion, compared with the *P. gingivalis* culture supernatants (Fig. 3).

TACE may exist in both cell-bound and secreted forms. Therefore, the inhibition of TACE secretion on heat treatment or TLCK treatment of *P. gingivalis*, does not exclude the possibility that the production of this protein may still be induced, but instead be accumulated in the cell-associated fraction because of inefficient shedding from the cell surface. To address this question, cell lysates were also collected and TACE levels were measured and compared with those in the corresponding culture supernatants. After 6 h of challenge with *P. gingivalis* it was found that extracellularly released TACE levels were consistently higher than cell-

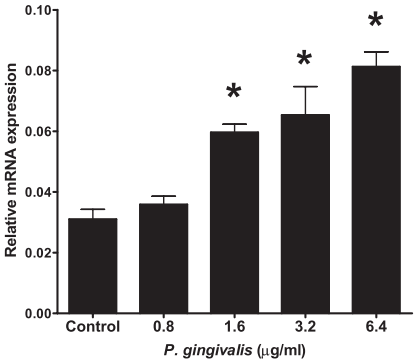

Fig. 1. Effect of *Porphyromonas gingivalis* on tumour necrosis factor-α converting enzyme (TACE) messenger RNA expression by Jurkat T-cells. Cells were cultured in the absence or presence of ascending protein concentrations of *P. gingivalis* culture supernatants for 6 h. Cell lysates were then collected, and TACE messenger RNA expression levels were measured by quantitative real-time polymerase chain reaction analysis, normalized against the expression levels of 18S ribosomal RNA. The results are expressed as the ΔCt formula. The bars represent mean ± SEM from three individual experiments. Asterisks indicate statistically significant difference compared with control group.

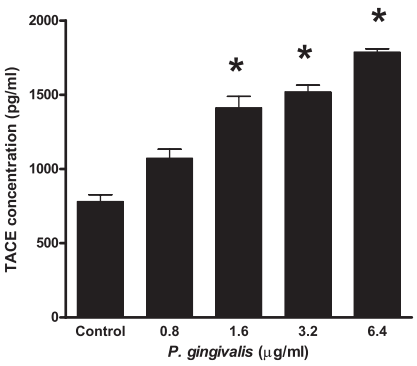

Fig. 2. Effect of *Porphyromonas gingivalis* on tumour necrosis factor-α converting enzyme (TACE) secretion by Jurkat T cells. Cells were cultured in the absence or presence of ascending protein concentrations of *P. gingivalis* culture supernatants for 6 h. The cell culture supernatants were then collected and analysed by enzyme-linked immunosorbent assay for TACE content. The bars represent mean ± SEM from three individual experiments. Asterisks indicate statistically significant difference compared with control group.

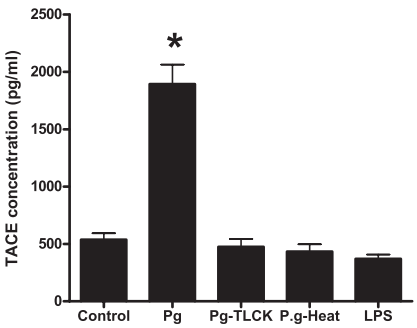

Fig. 3. Role of *Porphyromonas gingivalis* proteins, cysteine proteases and lipopolysaccharide (LPS) in tumour necrosis factor-α converting enzyme (TACE) secretion. Cells were cultured for 6 h in the absence or presence of 6.4 μg/ml untreated, heat-inactivated, or TLCK-treated *P. gingivalis* culture supernatants, or purified *P. gingivalis* LPS (1000 ng/ml). The cell culture supernatants were then collected and analysed by enzyme-linked immunosorbent assay for TACE content. The bars represent mean ± SEM from three individual experiments. Asterisks indicate statistically significant difference compared with control group.

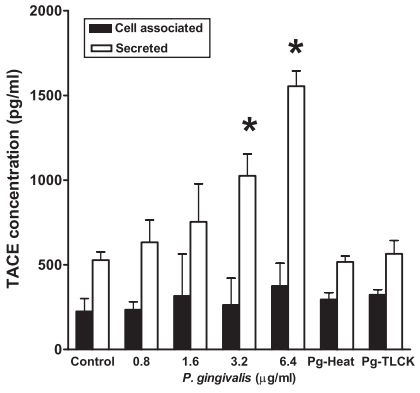

Fig. 4. Secreted vs. cell-associated tumour necrosis factor-α converting enzyme (TACE) levels in response to *Porphyromonas gingivalis*. Cells were cultured for 6 h in the absence or presence of ascending concentrations of *P. gingivalis*, heat-inactivated, or TLCK-treated *P. gingivalis* culture supernatants (6.4 μg/ml), or purified *P. gingivalis* lipopolysaccharide (LPS; 1000 ng/ml). The cell culture supernatants, as well as cell lysates were then collected and analysed by enzyme-linked immunosorbent assay for TACE content. The bars represent mean ± SEM from three individual experiments. Asterisks indicate statistically significant difference compared with control group.

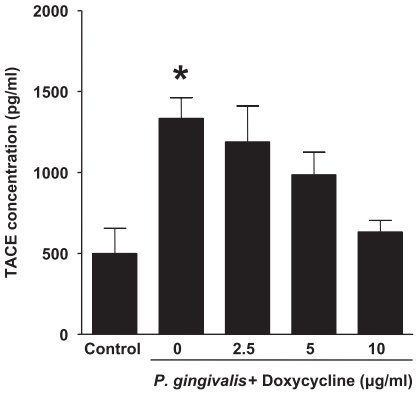

Fig. 5. Effect of doxycycline on *Porphyromonas gingivalis*-stimulated tumour necrosis factor-α converting enzyme (TACE) secretion. Cells were cultured for 6 h in the absence or presence of *P. gingivalis* culture supernatants (6.4 μg/ml), in combination with ascending concentrations of doxycycline (2.5–10 μg/ml). The cell culture supernatants were then collected and analysed by enzyme-linked immunosorbent assay for TACE content. The bars represent mean ± SEM from three individual experiments. Asterisks indicate statistically significant difference compared with control group.

associated levels. Interestingly, no statistically significant differences ($P > 0.05$) were observed in cell-associated TACE levels, between *P. gingivalis*-challenged and control cell cultures. Importantly, neither heat treatment, nor TLCK treatment had significant effects on cell-associated TACE levels, compared with either untreated *P. gingivalis* or control, whereas secreted TACE was consistently inhibited (Fig. 4).

Finally, the potential effect of doxycycline on TACE secretion was further investigated because this pharmacological agent is known to possess anti-inflammatory properties. Jurkat cells were challenged for 6 h with *P. gingivalis* culture supernatants alone (6.4 μg/ml), or in combination with ascending concentrations of sub-antimicrobial doses of doxycycline (2.5–10 μg/ml). These concentrations were shown to be non-toxic to the cells (data not shown). It was found that the presence of doxycycline in culture caused a significant and dose-dependent reduction of TACE secretion (Fig. 5). Doxycycline alone (10 μg/ml) caused a reduction in basal levels of secreted TACE by 30%, which, however, did not prove to be significant.

Discussion

This is the first study to demonstrate that *P. gingivalis* stimulates the mRNA expression and production of the zinc-dependent metalloprotease TACE by host cells. This property adds up to the capacity of this periodontal pathogen to modulate the inflammatory processes. Although *P. gingivalis* can regulate the expression of several metalloproteases, TACE is particularly important because of its capacity to shed membrane-bound cytokines from host cells, thus perpetuating the inflammatory responses.

Since bacterial challenge has been shown to stimulate TACE production in other experimental systems (13, 31–33), we aimed here to further identify the *P. gingivalis* virulence factors responsible for this effect. Heat inactivation of *P. gingivalis* culture supernatants abolished its capacity to stimulate TACE secretion by Jurkat T cells, indicating that the responsible component is proteinaceous. The involvement of cysteine proteinases was then further investigated by chemically blocking their activity in *P. gingivalis* culture supernatants. This procedure resulted in abolishment of the capacity of *P. gingivalis* to stimulate TACE production, implicating the gingipains as the responsible proteinaceous components. Purified *P. gingivalis* LPS failed to stimulate TACE secretion by the cells. To this extent, it has been shown that the production of TACE in monocytes is not affected by *Escherichia coli* LPS (9, 34), but can be downregulated by *Salmonella typhimurium* LPS (12). However, it should be noted that Jurkat cells do not express Toll-like receptors 2 and 4 (35), and may therefore not be responsive to LPS.

The release of TACE from the cell surface may require the involvement of proteolytic processing (36, 37). Since inhibition of *P. gingivalis* cysteine proteases appears to be associated with a decrease in TACE secretion, it was postulated that these bacterial enzymes may act to shed the membrane-bound form of TACE, especially because it possesses a cysteine-rich extracellular domain. If this would have been the case, then inhibition of *P. gingivalis* gingipain activity would consequently result in the accumulation of TACE on the cell-associated fragment as the result of inefficient shedding. To address this possibility, the levels of both cell-associated and secreted TACE were measured and compared. The data showed that cell-associated levels of TACE were not affected by *P. gingivalis* challenge compared with control, indicating that TACE is mainly expressed in its secreted form in the present experimental system. Importantly, the abolition of *P. gingivalis* gingipains by TLCK treatment did not result in the accumulation of cell-associated TACE, compared with challenge with untreated *P. gingivalis*. Similarly, heat inactivation of *P. gingivalis* did not enhance the cell-associated TACE levels. Hence, these data disprove the hypothesis that gingipains may act to shed *P. gingivalis*-induced TACE from the cell membrane. These findings indicate that the upregulated secretion of TACE in response to *P. gingivalis* occurs through a regulatory mechanism independent of gingipain-mediated shedding from the cells. A potential explanation would be the activation of proteolysis-mediated intracellular pathways. To this extent, gingipains have been shown to stimulate protease-activated receptors, triggering in turn a cascade of processes which play important roles in the host inflammatory responses (38).

The global cytokine shedding activity of TACE has implications in a wide range of pathological processes, including cancer (19), respiratory infections (13), bacterial meningitis (32), rheumatoid arthritis (15), inflammatory demyelinating disorders of the peripheral nervous system (39) and periodontitis (21). Therefore, the inhibition of TACE constitutes a putative therapeutic target for drug development. One of the potential approaches for TACE inhibition is the administration of doxycycline (28). Doxycycline possesses anti-inflammatory properties, which are separate and distinct from its antimicrobial action. These

include the inhibition of cytokine and metalloprotease production (25, 40, 41), and clinical studies have indicated that doxycycline treatment can be beneficial in inflammatory diseases associated with excessive metalloprotease production (26, 42). In the present study, the effect of doxycycline on *P. gingivalis*-induced TACE production was also investigated. The results demonstrated that doxycycline dose-dependently inhibited TACE secretion in *P. gingivalis*-challenged cells, within a concentration range between 2.5 and 10 μg/ml. This sub-antimicrobial range of doxycycline concentrations has been reported in gingival crevicular fluid, gingival tissue, or plasma after oral administration of this drug, and is therefore of physiological relevance (43, 44). Therefore, the pharmacological effects of doxycycline on the inhibition of TACE production may justify further the role of this drug as an adjunctive treatment agent for periodontitis. The exact mechanism of the inhibition of *P. gingivalis*-induced TACE production by doxycycline is not clear. However, this may be attributed to the anti-inflammatory properties of doxycycline because TACE can be stimulated by proinflammatory cytokines (45), and doxycycline can inhibit bacterially-induced cytokine production by T cells (40).

The present work has demonstrated that *P. gingivalis* stimulates TACE secretion by T cells, and its gingipains appear to be responsible for this effect. However, their proteolytic activity is not required for cell-shedding and release of TACE. Therefore, gingipains may have a role in regulating the overall production of secreted TACE, rather than its release from the cell membrane. This does not exclude the possibility that other periodontal pathogens may well induce TACE by host cells, via differential pathways. Bacterial stimulation of TACE secretion by T cells may result in ample cytokine shedding in the periodontal tissues, enhancing and prolonging the inflammatory responses, which could lead to the establishment of a chronic inflammatory lesion. These findings are of potential significance to periodontal diseases, especially in light of recent clinical evidence demonstrating higher gingival crevicular fluid levels of this molecule in periodontitis (21).

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