Treponema denticola alters cell vitality and induces HO-1 and Hsp70 expression in porcine aortic endothelial cells

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Abstract *Treponema denticola* is an oral spirochete that is associated with periodontal disease and detected occasionally in extraoral lesions associated with systemic disorders such as cardiovascular diseases. The effect of specific bacterial products from oral treponemes on endothelium is poorly investigated. This study analyzed the ability of components of the outer membrane of *T. denticola* (OMT) to induce apoptosis and heat shock proteins (HO-1 and Hsp70) in porcine aortic endothelial cells (pAECs), compared with results obtained with classical pro-inflammatory lipopolysaccharide (LPS) treatment. Cellular apoptosis was detected when pAECs were treated with either OMT or LPS, suggesting that OMT can damage endothelium integrity by reducing endothelial cell vitality. Stimulation with OMT, similarly to LPS response, increased HO-1 and Hsp-70 protein expression in a time-dependent manner, correlating

with a rise in HO-1 and Hsp-70 mRNA. Collectively, these results support the hypothesis that *T. denticola* alters endothelial cell function. Moreover, our in vitro experiments represent a preliminary investigation to further in vivo study using a pig model to elucidate how *T. denticola* leaves the initial endodontic site and participates in the development of several systemic diseases.

Keywords *Treponema denticola* · Endothelial cells · Hsp70 · HO-1 · Apoptosis

Introduction

Periodontal disease consists of inflammation of the periodontal tissues in response to bacterial accumulation on teeth. Bacterial plaques cause inflammation of gingival tissues, which leads to the destruction of periodontal ligaments, resorption of alveolar bone, and ultimately to tooth loss (Loesche and Grossman 2001). Periodontitis is associated with polymicrobial infections that are caused principally by anaerobic bacteria (Socransky et al. 1998). Among the microbiota that have been identified in periodontal tissues, there are several spirochetes that belong to the genus *Treponema* (Chan and McLaughlin 2000). *Treponema denticola* plays an important role in the progression of periodontal disease in humans (Ding et al. 1996; Leung et al. 2002; Rosen et al. 1999a); the virulence of *T. denticola* is associated with several enzymes and cytolytic factors that reside in the outer membrane, such as the major surface protein (MSP) and the surface-expressed chymotrypsin-like protease complex (CTLP). These factors can induce host immune cells to produce and release molecular mediators that affect the breakdown of periodontal connective tissue (Uitto et al. 1988; Ding et al. 1996;

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Mathers et al. 1996; Fenno and McBride 1998; Miyamoto et al. 2006; Tanabe et al. 2008). Moreover, *T. denticola* also produces a cell-associated lipooligosaccharide which can mediate macrophage activation resulting in nitric oxide, TNF- α , and IL-1 β production (Rosen et al. 1999).

Oral microorganisms, including spirochetes, are frequently associated with systemic disorders such as cardiovascular disease (Beck and Offenbacher 1998). Recently, *T. denticola* has been detected in atherosclerotic plaques by FISH (Cavirini et al. 2005) and in the brain in Alzheimer patients (Riviere et al. 2002); in addition, the ability of spirochetes to escape from the initial endodontic site of infection and consequently, to invade different anatomical sites has been reported in a SCID mouse model (Foschi et al. 2006).

Collectively, these data suggest that treponemes have direct interactions with vessels. Considering that endothelial cells are the cellular elements that first come into contact with blood vessel contents, oral treponemes can interact with endothelial cells both in highly vascularized periodontal tissue and outside the periodontal site as consequences of *Treponema* high motility (Pietrantonio et al. 1988). Okuda et al. (2007) demonstrated that *T. denticola* activates human endothelial cells by inducing interleukin-8 and macrophage chemoattractant protein 1 expression, however, the effect of specific bacterial products from oral treponemes on endothelium is poorly investigated.

Vascular endothelial cells respond to harmful stimuli by activation of multiple strategies, in particular, heat shock protein (HSP) induction is a primitive cellular defense also activated by endothelial cells against endotoxin insult (Bernardini et al. 2005). Among HSPs, HO-1, also known as Hsp32, and the products of its enzyme activity perform important physiological functions in the vascular system, including protection of endothelial cells against apoptosis and reduction in inflammatory cytokine production and proangiogenic and antifibrotic effects (Dulak et al. 2008; Bernardini et al. 2009). Hsp70 acts as a survival factor and an apoptosis inhibitor (Buzzard et al. 1998) and promotes a protective cellular environment which might attenuate inflammatory disorders by reducing inflammatory cytokine expression (Ding et al. 2001), also through the inhibition of NF κ B (Guzhova et al. 1997; Chen et al. 2006; Zheng et al. 2008).

This is a preliminary study with the aim to investigate if a preparation of the outer membrane from *T. denticola* (OMT) is effective in altering vitality and HO-1, Hsp70 expression of a primary culture of porcine aortic endothelial cells (pAEC). Subsequently, an in vivo porcine model could be useful for studying the ability of *T. denticola* to escape from the initial endodontic site and to be associated with cardiovascular systemic disorders. In particular, in this study, we assessed the capacity of OMT to stimulate pAEC

apoptosis and Hsp70 and HO-1 expression, comparing these results with the pAEC response obtained with the classical pro-inflammatory lipopolysaccharide (LPS) from *Escherichia coli*.

Materials and methods

T. denticola growth and outer membrane preparation

The *T. denticola* strain ATCC 35405 was grown under anaerobic conditions in new oral spirochete medium as described by Chan et al. (1993). The outer membrane from *T. denticola* was prepared using the method described by Yang et al. (1998) with slight modification as follows. The bacteria were harvested at late stationary phase, washed, re-suspended in phosphate-buffered saline (pH 7.2) containing 10 mM MgCl₂, and extracted in Triton X-100. After repeated centrifugation, the supernatant was dialyzed for several days until the OMT precipitated, and it was centrifuged at 25,000 $\times$ g. The OMT was re-suspended in phosphate-buffered saline and stored frozen until used. OMT was run by standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% acrylamide gel. A Coomassie Blue stain was performed in order to compare the protein profile in OMT preparation respect to the protein profile of the whole cells. In order to analyze the monomeric form of individual polypeptides, selected outer membrane and whole *T. denticola* cell preparations were treated at 100°C in boiling water for 10 min.

Cell culture and treatments

pAECs were isolated and maintained as previously described (Bernardini et al. 2005).

All experiments were performed with cells from the third to the eighth passage.

Apoptosis induction

pAECs were grown in 8-well slide chambers (approximately 4 $\times$ 10⁴ cells per well; 354631 Becton-Dickinson). Outer membrane from *T. denticola* or LPS from *E. coli* 055:B5 (Sigma-Aldrich Co, St. Louis, MO, USA) was added (10 μ g/ml) to the pAEC culture medium for 24 h. Treated cells and relative controls were fixed with 1% paraformaldehyde.

Endotoxin-induced chromatin fragmentation was evaluated by the ApopTag Fluorescein in situ apoptosis detection kit (Intergen Purchase, NY, USA) according to the manufacturer's instructions. Briefly, cells were postfixed in pre-cooled ethanol/acetic acid (2:1, v/v) for 5 min and equilibrated with the appropriate buffer; then, cells were

incubated with terminal deoxynucleotidyl transferase enzyme for digoxigenin dNTP incorporation (30 min at 37°C) and after several washings with Dulbecco phosphate buffer saline (DPBS), finally incubated with anti-digoxigenin–fluorescein antibody. The reaction was stopped, and fluorescent anti-digoxigenin was added (30 min at room temperature in the dark). After several washings in DPBS, cells were counterstained with propidium iodide and observed under a Nikon epifluorescence microscope. At least a minimum of 200 cells were evaluated for each treatment.

HO-1 and Hsp70 expression

pAECs were placed in a flat-bottom 24-well plate (approximately 4×10^4 cells per well; 353813 Falcon, Becton-Dickinson) and grown until confluence. When cells reached the confluence, OMT or LPS (10 µg/ml) was added to the culture medium for 7, 12, 18, and 24 h. At each time point, treated cells and relative control cells were collected and stored at -80°C for analysis of HO-1 and Hsp70 expression.

Real-time PCR

Total RNA was isolated using the RNeasy Mini Kit 50 (Qiagen Sciences Inc, Germatown, MD, USA) and treated with RNase-free DNase set (Qiagen) according to the manufacturer's instruction.

RNA concentration was spectrophotometrically quantified (A_{260} nm), and its quality was determined by gel electrophoresis on 2% agarose gel. One microgram of total RNA was reverse-transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-RAD Laboratories Inc., California, USA) in a final volume of 20 µl. Swine primers [HO-1, Hsp70, hypoxanthine–guanine phosphoribosyltransferase (HPRT)] were designed using Beacon Designer 2.07 (Premier Biosoft International, Palo Alto, CA, USA). Their sequences, expected polymerase chain reaction (PCR) product length, and accession number in the EMBL database are shown in Table 1. Real-time quantitative PCR was performed in the iCycler thermal cycler (Bio-RAD) using SYBR green I detection.

A master mix of the following reaction components was prepared to the indicated end-concentrations at 1.5 µl forward primer (100 ng/µl), 1.5 µl reverse primer (100 ng/µl), 7 µl water, and 12.5 µl IQ SYBR Green Bio-RAD Supermix (Bio-RAD). cDNA (2.5 µl) was added to 22.5 µl of the master mix. All samples were performed in duplicate.

The real-time PCR program was: initial denaturation for 1 min and 30 s at 95°C , 40 cycles of 95°C for 15 s, and 60°C for 30 s, followed by a melting step with ramping from 55°C to 95°C at a rate of 0.5°C/s .

The housekeeping HPRT was used to normalize the amount of RNA. The expression of HO-1 and Hsp70 mRNA was calculated as delta C_T (HPRT C_T –HO-1 C_T or Hsp70 C_T).

Real-time efficiency for each primer set was acquired by amplification of a standardized cDNA dilution series. The specificity of the amplified PCR products was verified by melting curve analyses and an agarose gel electrophoresis.

Western blot

Cells were harvested and lysed in SDS solution (Tris-HCl 50 mM pH 6.8; SDS 2%, 5% glycerol). Protein content of cellular lysates was determined using a Protein Assay Kit (TP0300, Sigma-Aldrich Co). Aliquots containing 15 µg of proteins were separated on NuPage 4% to 12% Bis–Tris gels (Gibco-Invitrogen, Paisley, UK) for 50 min at 200 V. Proteins were then electrophoretically transferred onto a nitrocellulose membrane. Western blot analyses of Hsp70 and HO-1 were performed as previously described (Bernardini et al. 2005) using a mouse anti-Hsp70 monoclonal antibody (SPA 810, StressGen, Victoria, BC, Canada) and a rabbit anti-Hsp32 polyclonal antibody (SPA 896, StressGen).

In order to normalize Hsp70 and HO-1 protein expression on housekeeping protein, membranes were stripped and re-probed for the antibody against the housekeeping HPRT (1:250 sc-5274 Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). The relative protein content (protein of interest/HPRT) was expressed in arbitrary units.

Table 1 Sequences, expected PCR product lengths, and accession numbers in the EMBL database of the swine primers for Hsp70, HO-1, and the housekeeping gene hypoxanthine–guanine phosphoribosyltransferase (HPRT)

Gene	Sequence (5'–3')	Length (bp)	Acc. No.
Hsp70	For.: GTGGCTCTACCCGCATCCC Rev.: GCACAGCAGCACCATAGGC	114 bp	M29506
HO-1	For.: CGCTCCCGAATGAACAC Rev.: GCTCCTGCACCTCCTC	112 bp	NM_001004027
HPRT	For.: GGACAGGACTGAACGGCTTG Rev.: GTAATCCAGCAGGTGAGCAAAG	115 bp	AF143818

Fig. 1 SDS-PAGE analysis of the OMT preparation, before and after treatment at 100°C for 10 min (lanes 1 and 2, respectively), lanes 3, and 4 contain whole *T. denticola* cells, before and after treatment at 100°C for 10 min. Filled circle indicates the position of the monomeric MSP (approximately 53 kDa); filled triangle indicates the position of the monomeric CTLP (approximately 72 kDa); number sign indicates the typical double band of multimeric CTLP (approximately 95 kDa); asterisk indicates the position of the CTLP/MSP complex. The positions of molecular size standards (low-range SDS-PAGE standards; Bio-RAD) are shown in kilodalton (kDa) on the left

Statistical analysis

Each treatment was repeated three times. Data were analyzed using one-way ANOVA followed by the Duncan's post hoc test (SPSS version 13.0, SPSS Inc, Chicago, IL, USA). Data are presented as mean $\pm$ 2SEM, and a difference of at least $p < 0.05$ was considered significant.

Results

SDS-PAGE analysis of the OMT

SDS-PAGE analysis of the OMT extract and whole *T. denticola* cells is shown in Fig. 1; in OMT preparation after heat treatment (lane 2), the most relevant bands showed a molecular mass of 53 and of 72 kDa corresponding to the monomeric form of MSP and CTLP (Fenno and McBride 1998). In unheated samples (lanes 1, 3), a typical double band corresponding to multimeric CTLP (approximately 95 kDa) was detectable (Fenno et al. 1998) and a higher band, more intense in OMT preparation than whole *T. denticola* cells, ascribable to a CTLP/MSP complex was detected.

Apoptosis

The addition of OMT to pAEC cultures was effective in inducing a significant increase of apoptosis compared with control ($14.6 \pm 1.7\%$ vs $4.7 \pm 0.3\%$). The rate of apoptosis observed was similar to that induced by LPS treatment (13.8 ± 0.5 ; Fig. 2).

HO-1 and Hsp70 expression

The time of culture did not influence HO-1 and Hsp70 expression in control cells (data not shown). Consequently, we used the mean of all the control time points as control (C). OMT was effective in inducing the heat shock response (HSR), and this response was similar to that of LPS. In fact, OMT induced a significant, transient

Fig. 2 Effect of *T. denticola* outer membrane in pAECs: apoptosis levels after 24 h of exposure to OMT or LPS. **a** Apoptosis was assessed by TUNEL assay, and cells are counterstained with propidium iodide. Cells with green fluorescence are apoptotic; **b** percentage of

apoptotic cells. A minimum of 200 cells were evaluated for each treatment; data represent the mean $\pm$ 2 SEM of three replicates. Different letters indicate statistically significant differences ($p < 0.05$). C control, OMT *T. denticola* outer membrane, LPS lipopolysaccharide

Fig. 3 Effect of OMT (**a**) or LPS (**b**) HO-1 mRNA levels in pAECs. Data are presented as Δ Ct (HPRT Ct–HO-1Ct) $\pm$ 2 SEM of three replicates. C control, OMT *T. denticola* outer membrane, LPS lipopolysaccharide

increase in both HO-1 and Hsp70 either at mRNA or at protein levels.

HO-1 mRNA increased immediately after stimulation with OMT or LPS (Fig. 3), peaking at 7 h, then declined slowly but exceeded control levels until 24 h. This increase in HO-1 mRNA was accompanied by a persistent rise in HO-1 protein from 7 to 18 h (Fig. 4).

Hsp70 mRNA increased more slowly and less robustly than HO-1. Hsp70 mRNA reached a maximum at 12 h and declined, returning to control levels at 24 h in OMT and LPS-treated cells (Fig. 5). Hsp70 protein content reflected a

transient increase until 12 h, after which it sharply declined below control levels (Fig. 6).

Discussion

In the present study, we evaluated the effects of *T. denticola* on primary porcine aortic endothelial cells in comparison with results showing the ability of LPS from *E. coli* to induce apoptosis of pAEC. A similar apoptotic effect by the outer membrane extract of *T. denticola* was

Fig. 4 Effect of OMT (**a, c**) or LPS (**b, d**) on HO-1 protein levels in pAECs. **a, b** Representative Western blot of HO-1 and relative housekeeping HPRT are presented. **c, d** Data are expressed as arbitrary

units (AUs) and represent the mean $\pm$ 2 SEM of three replicates. Different letters indicate statistically significant differences ($p<0.05$). C control, OMT *T. denticola* outer membrane, LPS lipopolysaccharide

Fig. 5 Effect of OMT (a) or LPS (b) Hsp70 mRNA levels in pAECs. Data are presented as Δ Ct (HPRT Ct–Hsp70Ct) $\pm$ 2 SEM of three replicates. C control, OMT *T. denticola* outer membrane, LPS lipopolysaccharide

detected. In substance, *T. denticola* also perturbs the cell cycle of pAEC, leading to the activation of an apoptosis cascade, and this mechanism is a common target utilized by many pathogens (Lee et al. 2004). Furthermore, the ability of *Treponema* to induce apoptosis in endothelial cells confirms that observed in other cellular types, including porcine periodontal ligament epithelial cells (Leung et al. 2002) and human lymphocytes (Lee et al. 2004). Endothelium integrity is fundamental to avoiding the progression of several inflammatory diseases; Seinost

and colleagues (2005) hypothesized that the endothelial dysfunction that is associated with severe periodontitis is provoked by direct invasion of the vessel wall by oral pathogens or their bacterial products. Furthermore, a recent review on bacteriemias that have an odontogenic origin suggested that oral spirochetes, including treponemes, affect the fetus, possibly through fetoplacental passage (Parahitiyawa et al. 2009), thus corroborating the hypothesis that treponemes leave oral sites and enter the bloodstream.

Fig. 6 Effect of OMT (a, c) or LPS (b, d) on Hsp70 protein levels in pAEC. a, b Representative Western blot of HO-1 and relative housekeeping HPRT are presented. c, d Data are expressed as arbitrary units (AUs) and represent the mean $\pm$ 2 SEM of three

replicates. Different letters indicate statistically significant differences ($p < 0.05$). C control, OMT *T. denticola* outer membrane, LPS lipopolysaccharide

Although the evidence that the ability of *Treponema* to directly alter endothelial cell function is poorly investigated, our results suggest the possibility that treponemes exert a noxious effect on endothelial cells by altering cell vitality.

The capacity of *T. denticola* to perturb the endothelial quiescence was also evidenced by the induction of heat shock proteins. The HSR represents a highly conserved mechanism of defense against many physical and chemical stressors, including heat shock, osmotic stress, endotoxin stimulation, shear stress, and heavy metals (Hartl 1996; Morimoto et al. 1994). We previously showed (Bernardini et al. 2005) that LPS induces an HSR in pAECs by stimulating the synthesis of anti-inflammatory Hsp70 and HO-1 proteins. In the current study, we found that a preparation of outer membrane of *T. denticola* induces a similar HSR. The different kinetics of HO-1 and Hsp70 induction reflect the disparate roles of these heat shock proteins: Hsp70 prevents protein misfolding and promotes the proteolytic degradation of damaged proteins therefore, a reduction in Hsp70 could be due to functional protein consumption; while the robust and prolonged induction of HO-1 is consistent with a prosurvival significance.

Furthermore, the same kinetic trend of Hsp expression by both LPS and OMT could suggest a similar cellular pathway of induction. Further investigations are necessary to identify which components of *T. denticola* outer membrane are involved in endothelial cell stress response.

Although the pathogenicity of *T. denticola* in pig has not been demonstrated, our results showed the ability of *Treponema* to perturb porcine endothelial cell homeostasis. This preliminary in vitro investigation was necessary, in accordance with a reduction in the use of experimental animals, to design further additional studies using an in vivo porcine model, with the aim to elucidate how *T. denticola* escapes from the initial endodontic site of infection and invades different anatomical sites, thus contributing to the development of several diseases.

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