Treponema denticola Outer Membrane Enhances the Phagocytosis of Collagen-Coated Beads by Gingival Fibroblasts

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Human gingival fibroblasts (HGFs) degrade collagen fibrils in physiological processes by phagocytosis. Since *Treponema denticola* outer membrane (OM) extract perturbs actin filaments, important structures in phagocytosis, we determined whether the OM affects collagen phagocytosis in vitro by HGFs. Phagocytosis was measured by flow cytometric assessment of internalized collagen-coated fluorescent latex beads. Confluent HGFs pretreated with *T. denticola* ATCC 35405 OM exhibited an increase in the percentage of collagen phagocytic cells (phagocytosis index [PI]) and in the number of beads per phagocytosing cell (phagocytic capacity [PC]) compared with untreated controls. The enhancement was swift (within 15 min) and was still evident after 1 day. PI and PC of HGFs for bovine serum albumin (BSA)-coated beads were also increased, indicating a global increase in phagocytic processes. These results contrasted those for control OM from *Veillonella atypica* ATCC 17744, which decreased phagocytosis. The *T. denticola* OM-induced increase in bead uptake was eliminated by heating the OM and by depolymerization of actin filaments by cytochalasin D treatment of HGFs. Fluid-phase accumulation of lucifer yellow was enhanced in a saturable, concentration-dependent, transient manner by the *T. denticola* OM. Our findings were not due to HGF detachment or cytotoxicity in response to the *T. denticola* OM treatment since the HGFs exhibited minimal detachment from the substratum; they did not take up propidium iodide; and there was no change in their size, granularity, or content of sub-G1 DNA. We conclude that a heat-sensitive component(s) in *T. denticola* OM extract stimulates collagen phagocytosis and other endocytic processes such as nonspecific phagocytosis and pinocytosis by HGFs.

Spirochetes are commonly detected in very low numbers in healthy gingival crevices, but their population increases sharply in periodontal diseases. Not only are spirochetes related to the progression of periodontal diseases (4, 5, 7, 35), but their presence in health-associated plaque may be associated with increased susceptibility to the development of periodontitis (32). They may also serve as prognostic markers for disease recurrence following treatment (36). Spirochetes are often found in direct contact with the sulcular epithelium lining the periodontal pocket and the junctional epithelium maintaining the epithelial attachment to the tooth (23). Therefore, they have an opportunity to contribute directly to the development of gingival lesions. In acute necrotizing ulcerative gingivitis and in advanced lesions of periodontitis, spirochetes may invade into the connective tissue (24, 33). Hence, they and their metabolic products or cellular fragments which penetrate the gingiva may also have an opportunity for direct contact with immunocompetent cells and cells which maintain connective tissue homeostasis, including fibroblasts (10).

*Treponema denticola* is the spirochete most frequently isolated from human periodontal pockets. Several cytopathic responses of fibroblasts have been documented following their contact with *T. denticola* (2, 3, 11, 41, 44). In vitro, late-stage cellular responses include formation of plasma membrane folds or blebs, rounding and shrinkage from the normally stellate shape, a reduction in proliferative capacity in some studies, and, ultimately, detachment of a subset of fibroblasts from the substratum. We have shown that *T. denticola* induces disruption of human gingival fibroblast (HGF) F-actin, its rearrangement into a perinuclear array, and the reduction in total F-actin content, especially in the ventral third of the cells (2, 46). We have also shown that *T. denticola* suppresses the inositol phosphate pathway and inhibits stretch-induced calcium transients as well as oscillations of calcium ions in resting cells (20, 46). These profound effects on the actin cytoskeleton and related signaling pathways would be expected to alter critical fibroblast functions like locomotion, which is important for effective wound healing, or phagocytosis, which is crucial for physiologic remodeling and wound repair in the periodontium. As the cytoskeletal changes were shown to be induced by outer membrane (OM) extracts as well as whole *T. denticola* cells, our specific aim was to determine the effects of *T. denticola* OM extract on collagen phagocytosis by HGFs. We were interested in collagen uptake because (i) collagen fibers are the primary protein component in the connective tissue matrix of the periodontium and are particularly important in providing structural stability due to their tensile strength (37) and (ii) a net loss of gingival connective tissue collagen is characteristic of periodontal diseases (27).

**MATERIALS AND METHODS**

**Culture conditions and preparation of OM extracts.** *T. denticola* type strain ATCC 35405 stock cultures were maintained in a complex spirochete broth medium containing brain heart infusion, tryptic peptone, yeast extract, and volatile fatty acids, supplemented with 2.0% rabbit serum (8). This strain has been shown to stimulate F-actin rearrangement, plasma membrane blebbing, and degradation of endogenous fibronectin in HGFs (2, 10, 11, 46). OM extract was prepared by a modification of the detergent extraction method of Penn et al. (31), as we have previously described (46). Briefly, the bacteria were harvested at late stationary phase, washed twice in 0.01 M phosphate-buffered saline, pH 7.2 (PBS), resuspended, and dispersed uniformly in PBS containing 10 mM MgCl2 at a ratio of 10 ml of PBS per g (wet weight) of bacteria. After Triton X-100 (Surfact-Amps X-100; Pierce, Rockford, Ill.) extraction and repeated centrifugation, the supernatant was dialyzed against distilled water (molecular mass cutoff, 50 kDa; Spectra/Por; Spectrum, Houston, Tex.) for several days until the OM precipitated. The contents of the dialysis tubing were centrifuged at 25,000 × g for 45 min at 4°C. The pellet was then resuspended in distilled water to the predialysis volume and stored at −70°C until used. The dry weight con-

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The OM extract was tested for peptidase activity against the chromogenic peptide substrate 7-amino-4-methylcoumaryl-7-amido-4-methylcoumarin (AMCA; Molecular Probes, Eugene, Oreg.) as a standard, indicated a protein concentration of about 700 μg/ml in the OM extract. For all experiments except dose-response experiments, a dispersed suspension of 0.19 ng (dry weight) of OM/HGF cell was used. This corresponds to a concentration of 0.084 ng of protein/HGF cell and 0.31 × 10^(-5) ng of chromogenic activity/cell. In some experiments the OM extract was preheated at 60°C for 30 min or at 100°C for 10 min.

Veillonella atypica was chosen as a control since this oral bacterium is gram negative and is not generally considered a periodontal pathogen. Previous investigations in our laboratory have also shown that sonicated extracts of V. atypica, in contrast with those of three species of periodontal pathogens, do not affect collagen synthesis in an osteogenesis model (25). V. atypica ATCC 17744 was cultivated in ATCC medium 188 containing Trypsitace, yeast extract, sodium lactate, sodium thio-glucose, Tween 20, glucose, and distilled water adjusted at pH 7.5. The OM extract was prepared by the method used for T. denticola. V. atypica OM extract had a dry weight of 0.72 μg/ml, a protein concentration of 200 μg/ml, and no detectable SAAPNA-degrading activity.

Fibroblast culture. Primary explant cultures of HGFs were derived from biopsies of normal gingiva as described elsewhere (1). Flow cytometry experiments were conducted on cells grown to confluence in six-well plates (Corning Glass Works, Corning, N.Y.) obtained as follows. Confluent cells in T-75 flasks were trypsinized with 0.01% trypsin. The cell concentration was determined by electronic particle counting (Coulter Counter ZM; Coulter Electronics of Canada Ltd.), and the HGFs were plated at a concentration of 3 × 10^5 cells per well.

The cells were grown until confluent at 37°C in a humidified 5% CO_2 incubator in alpha minimal essential medium (α-MEM) supplemented with 10% (vol/vol) antibiotic solution (penicillin G [145 U/ml; Sigma], gentamicin sulfate [50 μg/ml; Gibco BRL, Grand Island, N.Y.], and amphotericin B [0.25 μg/ml; Gibco BRL] in α-MEM) and 15% (vol/vol) heat-inactivated fetal bovine serum (FBS; ICN Biomedicals Inc., Costa Mesa, Calif.). Cells between passages 3 and 12 were used for all experiments.

To test the effect of OM extract on specific as well as nonspecific bead phagocytosis, collagen type I (Co-I)- and BSA-coated beads were included (Fig. 1). Coated beads were prepared by the method of Lee et al. (22). Briefly, 80 μl of yellow-green fluorescence polystyrene microbeads (2.0 μm in diameter; excitation maximum = 490 nm; emission maximum = 515 nm; catalog no. L.5301; Molecular Probes, Eugene, Oreg.) were incubated for 10 min at 37°C in 1 ml of an acidic bovine collagen solution (Vitrogen 100; 3.0 mg/ml, catalog no. L5301; Molecular Probes, Eugene, Oreg.) at pH 7.4. The OM extract was prepared by the method used for T. denticola.

RESULTS

Statistical analysis. Each experiment included triplicate samples. Means and SE were calculated for group comparisons within and among experiments. The experiments were repeated at least two or three times except for the pinocytosis assay with the consecutive OM-LY treatment and the dose-response experiments for V. atypica OM extracts, which were done once. For comparisons between control and OM-treated HGFs, the percent difference between corresponding samples within each experiment was calculated. For comparison of key variables, probability values cited in the text were based on mean percent differences from several independent experiments. Student’s t-test for unpaired samples was used to test significance in most cases. Analysis of variance was used for analysis of multiple samples for the heated OM extract experiment. A P value of <0.05 was set as a standard for statistical significance. Results shown represent examples of the repeated experiments rather than an average.

T. denticola increases HGF collagen phagocytosis. OM treatment of HGFs induced a consistent, statistically significant, concentration-related increase in collagen-coated bead uptake (Fig. 1) shows results of a representative experiment; n = 3). The average of 11 independent experiments showed that the overall mean untreated HGF uptake of collagen-coated beads was increased from 20.4% ± 1.9% to 61.1% ± 3.6% (mean PI ± SE) following T. denticola OM challenge, a difference of...
OM challenge, an increase of 357% (P < 0.00001). In addition to the increase in the percentage of cells ingesting the beads, T. denticola OM challenge induced a statistically significant increase in the number of beads taken up per cell (Fig. 2, inset). Uptake of collagen-coated beads increased from 155 ± 3 to 230 ± 6 fluorescence units (mean PC of three independent experiments ± SE) following T. denticola OM challenge, an increase of 48% (P < 0.001). This finding indicates that OM challenge induces an increase in the phagocytosis phenotype as well as the phagoctytic capacity of HGFs. The increase observed in PI (Fig. 3 and 4) and PC (data not shown) of collagen-coated beads varied directly with the duration of incubation with OM. An exposure of HGF to T. denticola OM as brief as 15 min induced an obvious increase in the PI compared with controls.

To determine whether the observed phagocytic increase was transient, two identical sets of samples were challenged in parallel with OM extract. After the cells were washed, one set was tested immediately for PI (group 1), and the other was incubated overnight in serum-free α-MEM (to prevent cell division) and then tested for the PI the following day (group 2) (Fig. 4). The OM-induced enhancement was increased irreversibly and persisted for at least 16 h, even after a single 15-min OM challenge.

Increased BSA-coated bead uptake, a test of specificity. To determine whether the T. denticola OM-mediated enhancement was due to a general increase in the phagocytosis of protein-coated beads, cells were incubated with BSA-coated beads, which are generally taken up nonspecifically in very low numbers by HGFs (19). OM challenge to HGF monolayers induced a consistent and significant concentration-related increase in BSA-coated bead uptake, which was also persistent for at least 16 h (Fig. 1 and 4). The untreated control HGF uptake of BSA-coated beads was increased from 6.5% ± 0.9% to 29.7% ± 3.8% (mean PI of eight independent experiments ± SE) following T. denticola OM challenge, an increase of 357% (P < 0.001). Analysis of the mean number of BSA-coated beads ingested per cell revealed a dose-response increase smaller than that for the PC of collagen-coated beads (Fig. 2, inset). Time course experiments revealed a positive, linear relationship between the duration of OM incubation and BSA-coated PI (Fig. 3 and 4) and PC (data not shown). The OM-induced specific and nonspecific phagocytic enhancement was evidently an early-onset, long-lasting response.

Increased LY uptake. LY was used to test the effect of T. denticola OM extract on pinocytosis in contrast to the uptake of relatively large particles (~2-μm diameter). A time course study of LY incubation yielded a positive concentration response for accumulation of dye in control cells. Coincubation of HGFs with LY and T. denticola OM simultaneously yielded a significant, saturable, time-related enhancement of LY accumulation (Fig. 5A). In contrast, a 1-h pretreatment with T. denticola OM followed by washing and LY exposure did not seem to affect the subsequent fluid-phase endocytosis activity of HGFs (Fig. 5B).

![Graph showing concentration-related effect of T. denticola OM on HGF-OM specific (Co-I) and nonspecific (BSA) bead uptake.](http://iai.asm.org/)
**V. atypica** OM decreases collagen but does not affect BSA-bead uptake. To test whether the HGF phagocytosis phenotype can be enhanced by an OM extract of a bacterium other than *T. denticola*, the OM extract of *V. atypica* ATCC 17744 was used. In contrast to the *T. denticola* OM extract, *V. atypica* OM extract induced a significant decrease in the collagen-coated bead PI (Fig. 6A). The mean untreated control HGF uptake of collagen-coated beads decreased by 47% (P < 0.001) following *V. atypica* OM challenge (three independent experiments). In fact, *V. atypica* OM concentrations up to four times the standard concentration (0.192 ng/cell) yielded a concentration-dependent decrease in HGF uptake of collagen-coated beads (Fig. 6B). *V. atypica* OM extract did not induce a significant change in the PI for BSA-coated beads (Fig. 6A). The mean uptake of BSA-coated beads decreased by 19% following *V. atypica* OM challenge (P > 0.05, mean change over three independent experiments). Propidium iodide staining indicated that the *V. atypica* OM challenge did not induce HGF cell death (data not shown). These findings indicate that the *T. denticola* OM-enhanced BSA- and collagen-coated bead uptake by HGFs is not a general phenomenon typical for any bacterial OM extract.

**Heat sensitivity of *T. denticola* OM extract activity.** At the concentrations used here, a 1-h *T. denticola* OM extract challenge increased collagen-coated bead uptake 3-fold and BSA-coated bead uptake 4.5-fold. Heating at either 60°C for 30 min or 100°C for 10 min completely abolished the OM enhancement of both collagen- and BSA-coated bead uptake (data not shown). Lipopolysaccharides (LPS) are known to be stable at these conditions. Heating the OM at 60°C for 30 min should denature most proteins, yet the extract retained greater than 65% of its SAAPNA-degrading activity, as in our previous studies (20, 46). The SAAPNA-degrading activity was reduced to <10% of the control level after boiling of the OM extract for 10 min. The results suggest that the PI increase was mediated by a heat-sensitive component(s) of the OM, probably neither LPS-like molecules nor the native chymotrypsin-like protease.

**Cell viability.** Preliminary studies led us to choose an OM concentration of 0.192 ng/cell, as it seemed to induce minimal HGF detachment. Indeed, several experiments confirmed this finding. The mean cell loss after 60 min *T. denticola* OM challenge was only 11.4% ± 4.8% (mean loss over 14 independent experiments), which is comparable to the cell loss under control conditions (2).

By light microscopy, the HGFs appeared to be morphologically unchanged after *T. denticola* OM treatment (data not shown). Treatment at 0.192 ng/cell for 15 and 30 min did not change the HGF shape even after moderately vigorous washes. After 60 min of treatment, the cells appeared unperturbed, but following the wash, a subset of the cells did lose some of their
lateral contacts. Nevertheless, the shrinkage was minimal and rarely did the cells appear completely rounded.

Flow cytometry analysis of cell size and cytoplasmic granularity in cell suspensions showed that these parameters were not significantly different after T. denticola OM treatment. Several other findings indicated that the attached cells remained viable. A set of subconfluent cells, which were treated for 1 h with various concentrations of OM, washed and reincubated in serum-supplemented growth medium, proliferated normally (25 to 40% confluence at the time of OM treatment to 100% confluence several days later [data not shown]). Further, T. denticola OM-challenged cells did not take up propidium iodide, unlike the positive control ethanol-treated cells. Flow cytometry analysis of sulforadamine-DAPI-stained cells showed that OM-treated HGFs had similar proportions of cells in the stages of the cell cycle as the control HGFs (Fig. 7). There were very few cells with DNA content less than that of the G1-phase cells. As substantial proportions of cells in the sub-G1 peak would indicate dying cells with reduced DNA content, this finding suggests that the OM challenge at the concentrations used did not induce apoptotic HGF cell death.

Effect of cytochalasin. The involvement of actin in T. denticola OM-stimulated phagocytosis was determined by using cytochalasin D, an actin cytoskeleton-disrupting agent (6). At a concentration of cytochalasin D which disrupted actin stress fiber arrangement (indicated by fluorescein isothiocyanate-conjugated phalloidin staining), cytochalasin D strongly inhibited collagen-coated bead phagocytosis in controls but did not affect the BSA-coated bead uptake (Fig. 8). Cytochalasin D treatment greatly reduced uptake of both the T. denticola-enhanced collagen- and BSA-coated beads (Fig. 8). These results were not due to cytochalasin D-induced toxicity since the cells were not stained by propidium iodide.

DISCUSSION

We have provided clear evidence that exposure of HGFs to the OM of T. denticola causes rapid enhancement of their phagocytosis of protein-coated beads. At the concentration used, there was minimal cell detachment and cytotoxicity; the control, T. denticola-treated, and V. atypica-treated cells all showed negligible uptake of propidium iodide. Moreover, cell cycle analysis showed no increase in two of the hallmarks of apoptosis,
T. denticola ENHANCES COLLAGEN PHAGOCYTOSIS

T. denticola OM-induced enhancement of bead uptake. HGFs were counted and exposed to CIM tum after challenge with whole skeletal rearrangement but remaining attached to the substratum after challenge with whole T. denticola cells are viable, as measured by lactate dehydrogenase release, propidium iodide staining, colony formation, and limiting dilution assays (2, 9, 43). They also found rapid transport of T. denticola chymotrypsin-like protease into membrane-bound vesicles (43). They also found rapid transport of T. denticola chymotrypsin-like protease into membrane-bound vesicles (43). They also found rapid transport of T. denticola chymotrypsin-like protease into membrane-bound vesicles (43).

Although the chymotrypsin-like protease of T. denticola is considered a major virulence factor (15, 42) and is implicated in HGF detachment from the extracellular matrix and the increased permeability of epithelial barriers (2, 21, 43), the relative susceptibility of its SAAAPN-degrading activity at 60°C argues against its significance in phagocytosis enhancement. Heat sensitivity also implies that LPS-like components are insignificant. Since the OM extract of gram-negative V. atypica actually decreased collagen phagocytosis, it is unlikely that LPS would mediate the phagocytosis enhancement in the case of T. denticola. Our results agree with previous findings that early events in the response of HGF to T. denticola OM, like the suppression of calcium and inositol phosphate pathways, are mediated by heat-sensitive components, probably nonproteolytic proteins (20, 46).

**Hypothesis for the mechanism of OM enhancement of bead uptake.** T. denticola OM induced a prolonged enhancement of both specific and nonspecific bead uptake, as well as a transient increase in pinocytic activity. It is unlikely that the pinocytic and phagocytic enhancement were due to the same mechanism(s). Pinocytosis is a constitutive and spontaneous event occurring in most eukaryotic cells by internalization of plasma membranes as vesicles, probably involving clathrin-coated and non-clathrin-coated pits (34, 38). In contrast, phagocytosis of large particles is more specific, involving activated receptors and transmembrane signaling to initiate particle engulfment (13, 19, 22). The LY accumulation was probably not due to T. denticola OM permeabilization of the HGF plasma membranes. Previous work indicated that HGF cells loaded with the fluorochrome fura-2 showed no significant dye leakage following T. denticola OM treatment at an OM concentration higher than the one used in this study (20). Moreover, examination of the HGFs by fluorescence microscopy found bright yellow fluorescence distributed as small discrete points over the entire cell surface (not shown), which is suggestive of uptake into pinosomes rather than through pores or unrestricted channels. Irrespective of the mechanism of pinocytic enhancement, we hypothesize that the increased pinocytic activity may mediate the uptake of the OM or fragments of the OM, which could lead to the observed persistence of bead phagocytosis.

As collagen internalization probably involves a functional actin cytoskeleton (13, 19), some of the observed phagocytosis enhancement may be explained by effects on actin-dependent processes. Cytochalasin D, an actin filament-disrupting agent (6), inhibited the uptake of collagen-coated beads by control HGFs, which is in agreement with previous work on the effects of similar concentrations of various cytochalasins on collagen uptake (12–14, 19), but it did not affect BSA-coated bead ingestion. In T. denticola OM-pretreated HGFs, cytochalasin D caused a significant inhibition of the phagocytosis enhancement of both collagen- and BSA-coated beads. This was evidently not due to cytotoxicity following the prolonged (up to 4-h) exposure of HGFs to cytochalasin D since propidium iodide staining of all samples indicated no dye uptake. Therefore, the OM-mediated enhancement of both collagen and BSA phagocytosis depended on a functional actin cytoskeleton. This is a significant finding, since previous work has shown that T. denticola OM disrupts cortical actin and stress fiber organization and decreases the total filamentous actin content (9, 10, 43, 46). We did not determine whether actin was altered during the phagocytosis assay. Evidently, there were sufficient numbers of actin oligomers and barbed ends in subcortical sites to start filament assembly, to permit binding to integrin receptors, and to initiate bead internalization by the OM-treated HGF. In what is perhaps an analogous example, there is some precedent for cytochalasin D, at a concentration lower than the one we used, to induce actin disruption but at the same time increase the uptake of bacteria in enterocytes (45).

Our results provide a novel demonstration suggesting that a spirochete associated with periodontitis may deregulate endo-

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**FIG. 8.** Cytochalasin D treatment of HGFs abolished the T. denticola OM-induced enhancement of bead uptake. HGFs were counted and exposed to CIM treatment (Cont) or T. denticola OM (0.192 ng of OM/HGF for 1 h). Cells were washed free of the extract, recounted, and then incubated in either α-MEM or 1 μM cytochalasin D (Cyt D) for 1 h. PI is expressed as mean ± SE (three independent samples of 10,000 cells each). *P < 0.01; **P < 0.0001 versus the corresponding non-cytochalasin D-treated group. Col, collagen.
cytotoxic and thereby enhance the intracellular uptake of collagen by gingival fibroblasts. This is significant in light of the fact that intracellular degradation of collagen has been proposed as the physiologic pathway for remodeling matrix and for wound repair rather than contributing to pathogenesis (30, 37). Perhaps our central finding, that the OM of a putative periodontal pathogen enhances collagen phagocytosis by HGFs, will provoke debate about whether bacterial stimulation of this physiologic pathway has relevance in the chronicity of periodontal diseases.

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