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INVESTIGATIVE REPORT

Treponema pallidum Induces Up-regulation of Interstitial Collagenase in Human Dermal Fibroblasts

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In this study we investigated the capability of *Treponema pallidum* to stimulate human dermal fibroblasts to produce interstitial collagenase (MMP-1), which is needed to degrade type I collagen, the most abundant component of the human dermis. When *T. pallidum* was added to human dermal fibroblast culture, both the amount of secreted MMP-1 and its mRNA levels were increased. Our results show that *T. pallidum* can stimulate host human fibroblasts to increase the synthesis of MMP-1, which may act as a virulence factor of the organism. ***Key words: T. pallidum; fibroblasts; interstitial collagenase.***

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Since its discovery almost a century ago, *Treponema pallidum* (*T. pallidum*) has evaded numerous attempts to discover its pathogenic features. Although the *T. pallidum* genome has recently been sequenced (1), the expectation that the analysis could open the door to understanding the pathogenic mechanism of this enigmatic organism has not yet been realized. Possible virulence factors include a family of 12 potential membrane proteins and several putative hemolysins but the genome contains neither the orthologs for any well-known virulence factors nor the secretory apparatus needed to deliver virulence factors into the host environment (2).

One of the virulence mechanisms of a pathogenic organism is to invade or destroy tissues in order to penetrate the anatomical barrier (3). In order to fulfill this task, it can be hypothesized that enzymes that can induce degradation of the connective tissue barrier are required for the manifestation of its pathogenicity. It is known that some microorganisms produce either cell-bound or extracellular enzymes needed to disrupt the connective tissue matrix directly (4). Collagen is the predominant component of skin and tendons and, at the same time, forms the scaffold of the major internal organs to give them their structure and strength. Collagen constitutes approximately 25 to 33% of the total protein in mammalian organisms (5) and any event that induces alteration of the homeostasis of collagen is likely to result in significant pathologic problems.

Interest in microbe-derived collagenases started toward the end of the 19th century when proteolytic clostridia were found to cause tissue putrefaction and, subsequently, extracellular enzymes of *Clostridium histolyticum* capable of digesting tendons were isolated (6). Since then, various pathogenic organisms, e.g. *Bacteroides* spp. (7), *Clostridium* spp. (8), *Pseudomonas aeruginosa* (9), *Treponema denticola* (10–12), *Treponema vincentii* (13), were found to produce various collagenases that are thought to be important virulence factors. The collagenases produced by the bacteria are presumed to release amino acids necessary for bacterial growth and/or to destroy natural defensive tissue barrier in order to facilitate bacterial spread (5). Further degradation of collagen from activation of latent mammalian collagenases by bacterial proteinases has also been suggested to be another mechanism by which bacteria can manifest their pathogenicity (14–16).

As *T. pallidum* has been found to lack a well-defined secretory apparatus (2), our goal was to determine whether *T. pallidum* could induce production of enzymes from other sources that can degrade the surrounding connective tissue. Since the most abundant component of the dermis is type I collagen, we have stimulated human dermal fibroblasts in culture with *T. pallidum* in order to find out whether this could directly influence the synthesised matrix metalloproteinase-1 (MMP-1).

MATERIAL AND METHODS

Culture of human dermal fibroblasts

Primary human dermal fibroblast cultures, established by explanting tissue specimens obtained from neonatal foreskin, were utilized in passages 3–8. The cell cultures were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum and 2 mM glutamine.

Subculture of T. pallidum and other treponemes

Subculture of *T. pallidum*, Nichols strain, obtained from the Centers for Disease Control (CDC; Atlanta, Georgia, USA) was done in specific pathogen-free, male New Zealand white rabbits weighing 2.5–3 kg. One milliliter of *T. pallidum* with a

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concentration of $2–3 \times 10^7$ treponemes/ml was inoculated into rabbit testicles and, after 7–10 days, *T. pallidum* was purified using Percoll density gradient centrifugation, as described elsewhere (17). *Treponema denticola*, biotype MRB, was purchased from the American Type Culture Collection (Rockville, MD, USA) and *Treponema phagedenis*, biotype Reiter, was obtained from the Centers for Disease Control (Atlanta, Georgia, USA).

Measurement of MMP-1 expression in culture supernatant

Confluent fibroblast dishes were trypsinized and the cell numbers were counted to obtain an average number of cells/dish and each confluent dish was exposed to various numbers of live *T. pallidum* ($3 \times 10^5 \sim 3 \times 10^8$ cells/well corresponding to $1 \sim 1 \times 10^3$ organisms/cell). Tumor necrosis factor-α (TNF-α, 10 ng/ml; Boehringer Mannheim, Indianapolis, IN, USA) was used as a positive control since it is known to up-regulate the expression of MMP-1 in human dermal fibroblasts. After 24 h incubation in an anaerobic incubator (CO_2 5%, H_2 10%, N_2 85%; Forma Scientific, Marietta, OH, USA), 0.5 ml culture medium (equivalent to about 50 μg total protein) was incubated with 2 μg anti-human-MMP-1 monoclonal antibody (Chemicon, Temecula, CA, USA) at 4°C. After 1 h, 50 μl Protein G Sepharose (Amersham, Uppsala, Sweden) was added and incubated at 4°C for another 3 h. The whole content was centrifuged for 2 min at 1×10^4 rpm to precipitate the Sepharose beads and the supernatant was discarded. The beads were washed 3 times with PBS and proteinase inhibitors (5 mM ethylenediaminetetraacetic acid, 5 mM phenylmethylsulfonyl fluoride) and the pellet was boiled in 20 μl sample buffer. After a brief centrifuge, 20 μl supernatant was run on a SDS-PAGE and transferred to a nitrocellulose membrane (Chemicon, Temecula, CA, USA). For the secondary antibody, we used goat $F(ab')_2$ anti-mouse immunoglobulins (Biosource, Camarhilo, CA, USA) and chemiluminescent detection using an ECL kit (Amersham, Uppsala, Sweden) was carried out.

Measurement of MMP-1 mRNA expression by Northern analyses

At the end of the 24 h incubation, cell cultures were subjected to isolation of total RNA, as previously described (18), and the purified RNA was quantitated spectrophotometrically. Equal amounts of RNA were fractionated in a 0.8% agarose gel containing formaldehyde and transferred to a nylon membrane (Hoeffer Scientific, MN, USA). The membrane with bound RNA was probed with ^{32}P-labeled cDNA probes for either inducible MMP-1 or constitutively expressed GAPDH, which served as an internal standard for mRNA loading onto filters. The $[^{32}P]$cDNA-mRNA hybrids were visualized by autoradiography, and the steady-state levels of mRNA were quantitated by scanning densitometry using a Phosphor Imager (BAS 2500; Fuji, Japan), setting the level of background value at 1. The level of MMP-1 mRNA expression was corrected for GAPDH mRNA expression and compared with the level of control MMP-1 mRNA expression which was set as 1.

Plasmid constructs

The human collagenase cDNA was a gift from Dr J. I. Kim (Pacific Research Institute, Suwon, Korea) and consisted of a 2 kb insert in a pSP64 vector. The insert was excised with Hind*III*-Sma*I* digestion and purified for use in hybridization. The human MMP-1 promoter/CAT vector was a gift from Dr Arthur Z. Eisen (Washington University School of Medicine, MO, USA). The MMP-1 promoter/luciferase (Luc) construct was generated by subcloning 2.3 kB *Hind*III/*Xho*I 5' flanking sequence of MMP-1 promoter gene from the MMP-1 promoter/CAT construct into *Sma*I/*Xho*I sites of pGL3-Basic.

Measurement of transcriptional activation of MMP-1 promoter/Luc construct by T. pallidum

For the transient transfection assay, NIH3T3 cells were plated at a density of 2×10^5 cells/35 mm dish. The following day, transfection was performed with 0.8 μg MMP-1 promoter/Luc construct and 0.2 μg pCMV-βgal (Clontech, Palo Alto, CA, USA). Transfection was performed for 3 h using Lipofectamin plus transfection reagent (GIBCO-BRL, Rockville, MD, USA) according to the manufacturer's instructions, and then fresh media containing *T. pallidum* were added. After 2 days, the cells were washed with phosphate-buffered saline and lysed in 200 μl reporter lysis buffer (Promega, Madison, WI, USA). Luciferase activities were measured using Luciferase Assay System (Promega, Madison, WI, USA) and normalized by the β-galactosidase activities to correct for the transfection efficiency.

Statistical analysis

Statistical analysis was done using the Kruskal–Wallis one-way analysis of variance and pairwise, multiple comparison test (Student–Newman-Keuls method) in the SigmaStat v2.0 program. Statistical significance was determined at the $p < 0.05$ level.

RESULTS

Stimulation of MMP-1 secretion from human dermal fibroblasts by T. pallidum

The secretion of MMP-1 by fibroblasts exposed to *T. pallidum* was determined by immunoprecipitation of the conditioned medium using an anti-MMP-1 monoclonal antibody. Human dermal fibroblasts were incubated until confluent, and varying numbers of *T. pallidum* were added to the culture medium for 24 h. TNFα, 10 ng/ml, was used as a positive control because it was already known to be a transcriptional activator for MMP-1 (19). At the end of the 24-h incubation, approximately 50% of the *T. pallidum* organisms were still motile. Immunoprecipitation of the culture medium (Fig. 1*A*) shows a single band of 52 kDa, which corresponds to the latent form of MMP-1. The results show that fibroblasts exposed to TNF-α and 3×10^8 *T. pallidum* show significantly increased secretion of MMP-1 as compared with the control.

T. pallidum increases the steady-state MMP-1 mRNA level in human dermal fibroblasts

The effect of *T. pallidum* on cellular steady-state levels of MMP-1 mRNA was determined by Northern blotting. Fibroblasts were incubated with increasing numbers of *T. pallidum* for 24 h, and total cellular RNA was extracted for Northern blot analysis. The result (Fig. 1*B*) shows that MMP-1 mRNA expression was increased significantly when fibroblasts were exposed to TNF-α, and 3×10^7 and 3×10^8 *T. pallidum*.

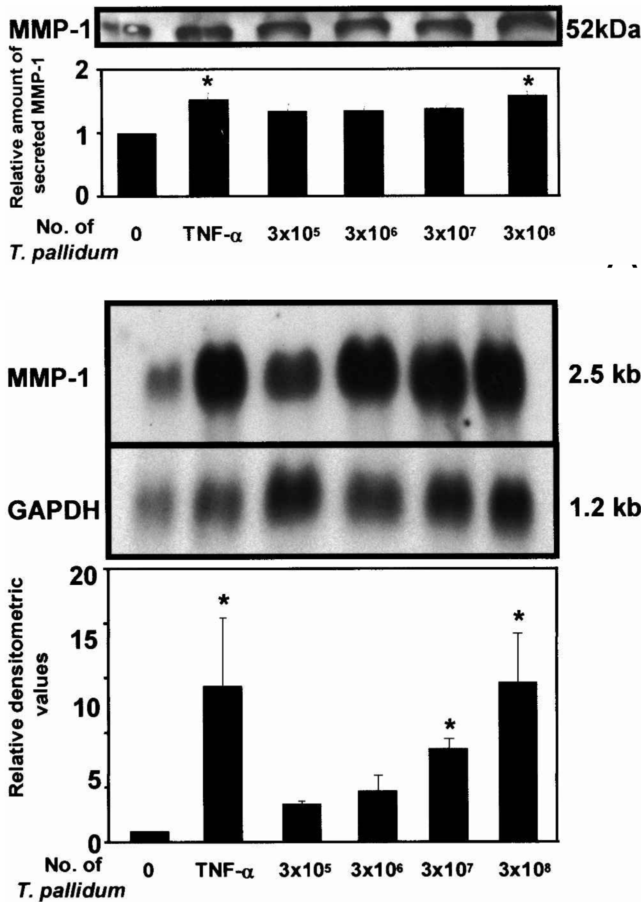

Fig. 1. Secretion of matrix metalloproteinase-1 (MMP-1) from human dermal fibroblasts is induced by *T. pallidum* protein. Neonatal foreskin fibroblasts were stimulated with varying numbers of *T. pallidum*/well and TNF-α (10 ng/ml) was used as a positive control. Twenty-four hours after stimulation, the amount of secreted MMP-1 in the medium was quantitated by immunoprecipitation (*A*) and MMP-1 mRNA expression of the fibroblasts by Northern blotting (*B*). The experiment was repeated three times and the density of each blot was measured with a densitometer, to create a bar graph. The autoradiographies of the first experiments are shown and each bar represents the mean ± standard deviation. A statistically significant increase when compared with the control ($p < 0.05$) is marked with an asterisk (*).

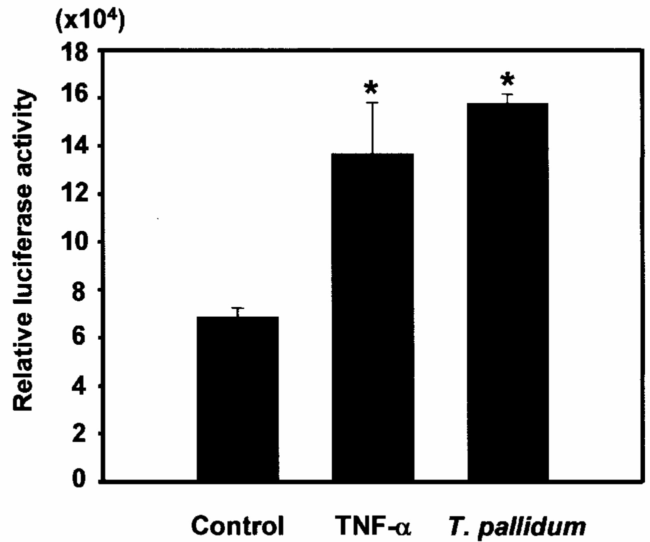

Fig. 2. T. pallidum stimulates matrix metalloproteinase-1 (MMP-1) promoter activity in NIH3T3 cells, which were transiently transfected with MMP-1 promoter/Luc construct and treated with TNF-α (10 ng/ml) or 3×10^8/well of *T. pallidum*. Luciferase activity was quantitated by a luminometer and each bar represents the mean ± standard deviation of 3 replicates. Asterisks (*) indicate statistical significance ($p < 0.05$) when compared with the control.

Effect of T. pallidum on the activities of MMP-1 promoter

To determine the level of *T. pallidum* action in up-regulating the MMP-1 synthesis in fibroblasts, the promoter gene of MMP-1 was subcloned into a pGL3 luciferase vector. Transient transfection and subsequent TNF-α and *T. pallidum* stimulation showed that MMP-1 promoter activities are stimulated by both TNF-α and *T. pallidum* (Fig. 2). The result shows that *T. pallidum* is capable of significantly up-regulating the MMP-1 gene at the transcriptional level.

DISCUSSION

Exactly how *T. pallidum* invades a human host has not yet been clarified, but *T. pallidum* is known to directly induce the expression of intercellular adhesion molecule-1 (ICAM-1) activity in human umbilical vein endothelial cells (HUVEC) (20) and also to promote lymphocytes and monocytes to adhere to HUVEC (21). *T. pallidum* has also been shown to stimulate human dermal microvascular endothelial cells to increase the expression of ICAM-1, vascular cell adhesion molecule-1 and E-selectin and, thereby, promote the adherence of T-lymphocytes (22). It has been suggested that binding of *T. pallidum* to human endothelial cells, which are capable of uptake and processing of antigens (23), might result in intracellular degradation and processing of the organisms (20). The process results in the production of biologically active lipoproteins, which are responsible for subsequent activation, and there have been reports of *T. pallidum* being identified intracellularly (24), especially in human endothelial cells (25, 26).

The purpose of our experiment was to determine whether *T. pallidum* could directly stimulate human dermal fibroblasts to produce MMP-1, which would degrade type I collagen, the major component of the dermis, and thereby assist *T. pallidum* migration in the collagenous dermis once it invades human skin. *T. pallidum* harvested from the rabbit testicular culture was purified, washed and aliquoted into the human dermal fibroblast cultures and incubated for 24 h in an anaerobic chamber.

Immunoprecipitation of the culture media with the monoclonal anti-human MMP-1 antibody showed that secreted MMP-1 from fibroblasts increased when *T. pallidum* was added directly to the cultured human fibroblasts. When compared with the positive control

induced by TNF-α, the amount of secreted latent form of MMP-1 significantly increased when 3×10^8 *T. pallidum* was added. This induction of MMP-1 coincided with the increase in its mRNA levels. MMP-1 mRNA expression was also found to be significantly increased in relation to the amount of *T. pallidum* added.

In an additional experiment to test for the difference in their ability to stimulate MMP-1 mRNA expression in human dermal fibroblasts, identical amounts of *T. pallidum*, *T. denticola*, and *T. phagedenis* were added to the confluent fibroblast cultures. Northern blot analysis showed that *T. denticola* and *T. phagedenis* did not have the ability to stimulate MMP-1 production in human dermal fibroblasts (data not shown).

T. denticola has been found to adhere to human gingival fibroblasts (27) and to degrade synthetic peptides and naturally occurring proteins but not type I collagen (28). *T. denticola* expresses cell-associated, chymotrypsin-like protease, capable of degrading type IV collagen, which is proposed to be the major contributory factor in its invasiveness (11). Our result coincides with the previous result (28) in that *T. denticola* did not have the capability directly to stimulate human dermal fibroblasts in up-regulating the MMP-1 mRNA expression, just like *T. pallidum*. *T. phagedenis*, a non-pathogenic organism, which was also unable to up-regulate the MMP-1 mRNA expression, as expected.

Since lipoproteins of *T. pallidum* are known to activate TNF-α at a transcriptional level (29), it is possible that *T. pallidum* could stimulate monocytes/macrophages to synthesize TNF-α and this in turn could up-regulate MMP-1 synthesis in fibroblasts. Our system did not allow any intermediate steps that include monocytes/macrophages, but the possibility of direct stimulation of fibroblast by *T. pallidum* to produce TNF-α has not been ruled out. However, we have shown that *T. pallidum* is capable of stimulating human dermal fibroblasts in the production of MMP-1, a fact that could contribute to further investigation into the pathogenicity of *T. pallidum*.

Through this study, we were able to determine that *T. pallidum* could induce and up-regulate MMP-1 secretion in human dermal fibroblasts and that this capability could be regarded as one of the novel pathogenic mechanisms of this organism.

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