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Substance P regulates the expression of matrix metalloproteinases and tissue inhibitors of metalloproteinase in cultured human gingival fibroblasts

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Background and Objective: Substance P may play a role in the pathogenesis of periodontal disease; however, its mechanisms of modulation are not clear. This study evaluated the effect of two concentrations of Substance P on the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in cultured human gingival fibroblasts.

Material and Methods: Fibroblasts were stimulated for 48 h with 10^{-4} or 10^{-9} M Substance P; untreated fibroblasts served as controls. The expression of MMP-1, -2, -3, -7 and -11 and of TIMP-1 and -2 was evaluated using real-time polymerase chain reaction and western blotting.

Results: There was a significant, concentration-dependent stimulatory effect of Substance P on MMP-1, -2, -3 and -7 and TIMP-2 gene expression ($p < 0.05$), and a probable effect on MMP-11 ($p = 0.06$). At the higher concentration (10^{-4} M Substance P), MMP-1, -2, -3, -7 and -11 and TIMP-2 showed the greatest up-regulation; at the lower concentration (10^{-9} M Substance P), MMP-1, -3 and -7 and TIMP-2 exhibited diminished up-regulation, with MMP-2 and -11 showing down-regulation ($p < 0.05$). Expression of TIMP-1 was not affected by Substance P ($p > 0.05$). Western blotting confirmed that Substance P up-regulated MMP-1, -2, -3 and -11 and TIMP-2. MMP-1, -3 and -11 and TIMP-2 showed greater up-regulation at the higher Substance P concentration and diminished up-regulation at the lower concentration. MMP-2 was up-regulated to a similar degree at both Substance P concentrations.

Conclusion: In gingival fibroblast cells, Substance P at the higher concentration (10^{-4} M) induced greater up-regulation of MMP-1, -3 and -11 and TIMP-2 expression, but at the lower concentration (10^{-9} M) induced diminished up-regulation, which may represent a mechanism for modulating periodontal breakdown.

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Periodontitis is an infectious disease characterized by connective tissue breakdown, mediated mainly by host-derived enzymes, such as matrix metalloproteinases (MMPs). The concentrations of MMP-1, -2, -3 and -7, tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), and others, are elevated during active periodontitis (1–6). However, the regulation of MMP expression during periodontitis is poorly understood.

A neurogenic component is involved in the pathogenesis of many inflammatory diseases, including periodontitis (7–11). Neurogenic inflammation is a protective mechanism, constituting a first line of defense and protecting tissue integrity. However, severe or prolonged noxious stimulation mediated by neurotransmitters or by mechanical stimuli may cause the inflammatory response to mediate injury (7). The main role of neurotransmitters is to traverse the synapse, interact with specific receptors and generate an electrical impulse. Alternatively, the neurotransmitter can be released into the extracellular matrix where it may exert a paracrine effect. Some neuropeptides are simple peptide neurotransmitters, and Substance P is a prominent member of the neuropeptide family of neurotransmitters. Substance P levels are significantly higher in the gingival crevicular fluid of patients with periodontitis than in healthy individuals (9–11), and Substance P may participate in the pathogenesis of periodontitis. It causes neurogenic inflammation (7,8), increases the immune and inflammatory responses (12–16) and inhibits the mitogenic capacity of gingival fibroblasts (17) and osteoblast cell differentiation (18).

The regulation of MMP expression during periodontitis apparently involves complex interactions between cell-surface receptors and the extracellular matrix, cytokines and growth factors (19). The expression of MMPs and TIMPs by gingival fibroblasts is regulated by hydrocortisone and epidermal growth factor *in vitro* (20,21). However, whether Substance P regulates the expression of MMPs and TIMPs is presently unknown. The present study examines the hypothesis that Substance P at a high concentration can up-regulate MMP and TIMP expression, and, conversely, that a low concentration of Substance P maintains a diminished expression of these proteins. Thus, we evaluated the effect of two different concentrations of Substance P on the regulation of expression of MMP-1, -2, -3, -7 and -11, and TIMP-1 and -2, in cultured human gingival fibroblasts.

Material and methods

Cells cultured in monolayer

The protocol employed was approved by the Ethics Committee of the School of Dentistry, University of São Paulo (2003).

Human gingival fibroblasts were cultured from healthy, uninflamed gingival explants removed during routine surgical procedures, as described previously (20,21), and used at the fifth passage. Briefly, explants were rinsed for 30 s in 70% alcohol and stored in Dulbecco's modified Eagle's medium (Sigma, St Louis, MO, USA) containing 10% antibiotic/antimycotic solution (Sigma). The explants were rinsed in phosphate-buffered saline (Sigma) containing 1% antibiotic/antimycotic solution, finely minced into portions of 1–2 mm^3, rinsed three times in phosphate-buffered saline and placed in tissue culture dishes. A thin layer of Dulbecco's modified Eagle's medium containing 10% antibiotic solution was added to the dishes and after 24 h the Dulbecco's modified Eagle's medium was supplemented. After 14–20 d, outgrowth cells were observed. After reaching approximately 70% confluence, the fibroblasts were harvested with trypsin (0.1% Trypsin + 0.1% EDTA, pH 7.2; Sigma) and subcultured.

For the experiments, 10^4 fibroblasts/mm^2 were plated onto 60-mm-diameter dishes. These cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Donor Bovine Serum; Gibco, Grand Island, NY, USA) and 1% antibiotic/antimycotic solution. When the culture reached approximately 80% confluence, the cells were washed in phosphate-buffered saline and incubated for 24 h in Dulbecco's modified Eagle's medium without fetal calf serum. Cell cultures were then exposed to Substance P (Sigma) at 10^{-4} or 10^{-9} M for 48 h in Dulbecco's modified Eagle's medium without fetal bovine serum. Untreated cells incubated without fetal calf serum served as controls. All experiments were performed in triplicate.

The explants and cells were incubated at 37°C in a humidified 95% air/5% CO_2 environment.

Sample preparation

After incubation, 100-μL aliquots of fibroblast culture medium were collected. Ten microlitres of protease inhibitor cocktail (Sigma) was added and the aliquots were stored at −70°C. The fibroblasts were then trypsinized, resuspended in 100 μL of phosphate-buffered saline, lysed by adding 0.9 mL of TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) and the RNA extracted according to the manufacturer's protocol.

The $A_{260} : A_{280}$ ratios were measured using a spectrophotometer and were always > 1.8.

Digestion of single- and double-stranded DNA was performed using DNase I Amp Grade (Deoxyribonuclease I, amplification grade; Invitrogen) according to the manufacturer's instructions.

The first-strand cDNA was synthesized. Briefly, 1 μg of RNA sample, 1 μL of random primer (Random Primers; Invitrogen), 1 μL of 10 mM dNTP Mix (Invitrogen) and distilled water to 13 μL were added to a microcentrifuge tube, heated to 65°C for 5 min and chilled on ice. Then, 4 μL of 5× First-Strand Buffer and 2 μL of 0.1 M dithiothreitol were added and incubated at 37°C for 20 s. One microlitre of Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen) was added and the tubes were incubated at 37°C for 50 min.

Real-time polymerase chain reaction

Alterations in the expression of mRNA for MMP-1, -2, -3, -7 and -11, and TIMP-1 and -2 genes were evaluated

using the real-time polymerase chain reaction (PCR) (7300 Real Time PCR System; Applied Biosystems, Foster City, CA, USA) and SYBR Green PCR Master Mix (Applied Biosystems). β-actin mRNA expression was used as a control gene to normalize the data. All real-time PCR assays were performed in 96-well optical plates (Applied Biosystems) using the following cycling parameters: 50°C for 2 min and 95°C for 10 min; PCR cycling for 40 cycles at 95°C for 15 s (denaturation), 60°C for 1 min (annealing) and a dissociation cycle (95°C for 15 s, 60°C for 30 s and 95°C for 15 s).

Oligonucleotide primers for MMP-1, -2, -3, -7 and -11 and β-actin were designed based on sequences in the GenBank database using Custom Primers-OligoPerfect™ Designer (Invitrogen). One sample of the PCR product obtained using each set of primers was sequenced (MegaBace; Amersham Bioscience, Sunnyvale, CA, USA). The primer sequences were deposited in GenBank (Table 1). Primer sequences for TIMP-1 and -2 were obtained from the literature (22,23).

Negative controls with SYBR Green PCR Master Mix (Applied Biosystems) and water were performed for all reactions.

To analyze gene expression, the mean (± standard deviation) CT values (the point at which the amplification curves cross the threshold line, which was adjusted to 0.9) were calculated for each set of reverse transcribed mRNA triplicates.

The difference between the expression of the target and the endogenous control gene (β-actin) was then calculated (ΔC_T), and the difference between target gene expression in the Substance P-treated cells and the control cells was computed ($\Delta\Delta C_T = \Delta C_T$ for Substance P-treated cells minus ΔC_T for control cells). The range of gene expression for each Substance P concentration was then estimated from the relation $2_T^{\Delta\Delta C}$.

Western blotting

Aliquots of fibroblast culture medium were centrifuged at 10,000 *g*. The protein concentration was estimated using a Protein 200 Laboratory-chip kit (Protein 200 Plus Assay; Agilent Technologies, Santa Clara, CA, USA) performed on an Agilent Bioanalyzer 2100. A 10-μg aliquot of each sample was then run on sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes (Hybond-P PVDF Membrane; Amersham Lifescience, Arlington Heights, NJ, USA) by eletroblotting. The membranes were blocked in 0.1% (v/v) Tween 20 and 5% (w/v) powdered milk in phosphate-buffered saline for 60 min at 4°C, rinsed briefly with phosphate-buffered saline/Tween 20, and incubated separately at a 1 : 1000 dilution of polyclonal anti-MMP-1, -2, -3, -7 or -11, or anti-TIMP-1 or -2 (NeoMarkers, Fremont, CA, USA) for 2 h at 37°C. The membranes were washed in phosphate- buffered saline/Tween 20 for 1 h and incubated in horseradish peroxidase-conjugated goat antimouse secondary antibody (diluted 1 : 10,000 in phosphate-buffered saline/Tween 20; Immun-Star Goat Anti-Mouse HRP Conjugate (Bio-Rad Laboratories, Hercules, CA, USA) for 60 min at room temperature. The blots were then incubated in Opti-4 CN substrate for 30 min (Bio-Rad, Hercules, CA, USA).

Images on the membranes were digitized (HP Scanjet 7400C Series Scanner – XPA; Hewlett-Packard Development, Palo Alto, CA, USA) and the intensity of each band was quantified using image analysis software (IMAGEJ 1.34s; National Institues of Health, Bethesda, MD, USA). The difference in band intensity between each target protein in the Substance P-treated cells and the untreated cells was computed.

Data analysis

A Kruskal–Wallis analysis of variance, followed by the Mann–Whitney *U*-test, were used to evaluate the effect of Substance P on the expression of

Table 1. Primer sequences (Forward, F; Reverse, R), product length and region, melting temperature (Tm) and accession number for matrix metalloproteinase (MMP)-1, -2, -3, -7 and -11, tissue inhibitor of metalloproteinase (TIMP)-1 and -2, and β-actin

Target gene	Primer Type	Primer Sequence	Product length	Region	Tm (°C)	Accession number
MMP-1	F	5′-ATGCTGAAACCCTGAAGGTG-3′	204	310–543	60	DQ399597
	R	5′-CTGCTTGACCCTCAGAGACC-3′				
MMP-2	F	5′AGGGCACATCCTATGACAGC-3′	186	1182–1367	60	DQ385623
	R	5′-ATTTGTTGCCCAGGAAAGTG-3′				
MMP-3	F	5′-GCAGTTTGCTCAGCCTATCC-3′	214	100–313	60	DQ399598
	R	5′-GAGTGTCGGAGTCCAGCTTC-3′				
MMP-7	F	5′-AAAGAGATCCCCCTGCATTT-3′	162	459–620	60	DQ399600
	R	5′-GTGAGCATCTCCTCCGAGAC-3′				
MMP-11	F	5′-TAGGTGCCTGCATCTGTCTG-3′	203	1969–2173	60	DQ399599
	R	5′-TGGCTTTGGAGGATAGCAGT-3′				
TIMP-1 (22)	F	5′-CTGTTGTTGCTGTGGCTGAT-3′	587	96–683	56.7	
	R	5′-TCCGTCCACAAGCAATGAGT-3′				
TIMP-2 (23)	F	5′-GCACATTACCCTCTGTGACTTC-3′	146	639–784	56.4	
	R	5′-AGCGAGTGATCTTGCACTCA-3′				
β-actin	F	5′-TGCGTGACATTAAGGAGAAGCT-3′	456	701–1157	56.7	DQ407611
	R	5′-TCCTGCTTGCTGATCCACAT-3′				

different target genes. The Mann–Whitney *U*-test was used to evaluate the effect of the different Substance P concentrations on the expression of each target gene. The significance level was set at 5%.

Results

Substance P had no significant effect on β-actin mRNA expression ($p > 0.05$), validating the usefulness of this gene as an internal control.

Human gingival fibroblasts constitutively expressed MMP-1, -2, -3, -7 and -11 and TIMP-1 and -2 genes after 48 h. Treatment with Substance P resulted in a significant difference in the ratio of up/down-regulation when comparing the different genes ($p < 0.01$).

There was a significant concentration-dependent effect of Substance P on MMP-1, -2, -3 and -7 and TIMP-2 gene expression ($p < 0.05$) and a possible effect on MMP-11 ($p = 0.06$). At the higher Substance P concentration (10^{-4} M), MMP-1, -2, -3 and -7 and -11 and TIMP-2 showed the greatest up-regulation; at the lower Substance P concentration (10^{-9} M), MMP-1, -3 and -7 and TIMP-2 exhibited diminished up-regulation, with down-regulation of MMP-2 and -11 ($p < 0.05$). MMP-3, -1, TIMP-2, MMP-2, MMP-7 and MMP-11 gene expression was markedly affected by Substance P-treatment: increases in RNA expression of up to 63-, 59-, 15-, 12-, 7- and 5-fold, respectively, were found ($p < 0.05$) (Fig. 1).

Expression of TIMP-1 was not significantly affected by Substance P ($p > 0.05$).

Western blotting confirmed an up-regulatory effect of Substance P on MMP-1, -2, -3 and -11 and TIMP-2. MMP-1, -3 and -11 and TIMP-2 showed higher up-regulation at the higher Substance P concentration and diminished up-regulation at the lower Substance P concentration. MMP-2 was up-regulated to a similar degree at both Substance P concentrations. Protein up-regulation was less than the up-regulation of the gene expression, except for MMP-11. MMP-7 expression was not found in either Substance P-treated or control cells (Fig. 2).

Discussion

The present study showed that Substance P regulates the expression of MMP-1, -2, -3 and -11 and TIMP-2 in a dose-dependent manner: the higher Substance P concentration (10^{-4} M) induced greater MMP-1, -3 and -11 and TIMP-2 up-regulation; the lower concentration (10^{-9} M) induced diminished up-regulation. Furthermore, expression of the TIMP-1 gene was not significantly affected by Substance P, and MMP-7 protein expression was not found in either Substance P-treated or control cells.

In this study, we examined the effect of two Substance P concentrations on MMP and TIMP expression: the higher concentration was similar to those described during inflammation and the lower concentration was below those reported for inflammation and may represent a physiological concentration. Substance P is present at very low levels ($< 10^{-8}$ M) in the serum, with the concentration increasing during neurogenic inflammation (24) and in periodontitis compared with the healthy periodontium (9–11). Hanioka *et al*. noted that the Substance P concentration increases with greater probing depths, reporting a mean level of 6.4×10^{-9} M (range: 0–2.84×10^{-7} M) (10). Lundy *et al*. showed that the Substance P concentration in gingival crevicular fluid decreases from 1.4×10^{-4} M to 2.4 10^{-5} M at sites with periodontitis after periodontal treatment (9). In the present study, the higher Substance P concentration (10^{-4} M) up-regulated MMP-3, MMP-1, TIMP-2, MMP-2, MMP-7 and MMP-11 RNA expression up to 63-, 59-, 15-, 12-, 7- and 5-fold, respectively. In contrast, the lower Substance P concentration induced diminished up-regulation or down-regulation. Thus, because the Substance P concentration is significantly higher during periodontitis than in the healthy periodontium (8–11), Substance P may be involved in the pathogenesis of periodontitis by regulating MMP and TIMP expression. Corresponding protein up-regulation was not as great as gene up-regulation, except for MMP-11. Western blotting confirmed that Substance P had an up-regulatory effect on MMP-1, -2, -3 and -11 and TIMP-2. The higher Substance P concentration produced the greatest

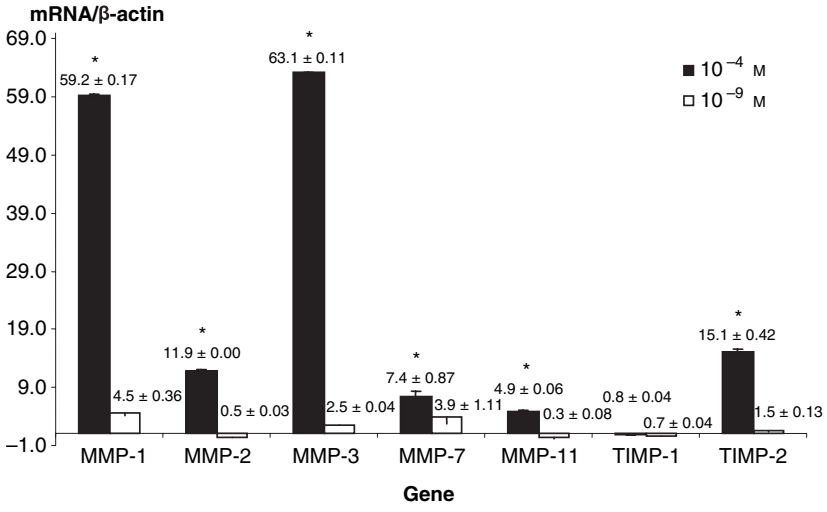

Fig. 1. Effect of Substance P on the mRNA expression of matrix metalloproteinase (MMP)-1, -2, -3, -7 and -11 and tissue inhibitor of metalloproteinase (TIMP)-1 and -2 genes in gingival fibroblasts as detected by semiquantitative, real-time polymerase chain reaction. The values (mean ± standard deviation) represent the range of gene expression at each Substance P concentration (10^{-9} and 10^{-4} M) estimated using the expression $2^{\delta\delta C}_{T}$ ($\Delta\Delta C_T = \Delta C_T$ for Substance P-treated cells minus ΔC_T for control cells; ΔC_T = expression of the target gene minus the β-actin gene). Values greater than 1 indicate up-regulation, whereas values less than 1 indicate down-regulation. *$p < 0.05$, comparing the two different Substance P concentrations for each gene using the Mann–Whitney *U*-test.

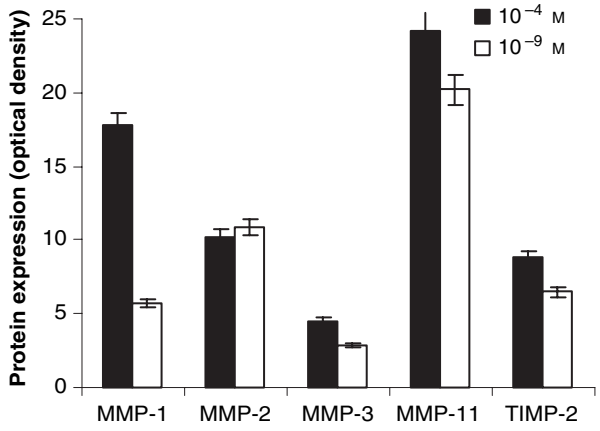

Fig. 2. Effect of Substance P on the expression of matrix metalloproteinase (MMP)-1, -2, -3, and -11 and tissue inhibitor of metalloproteinase (TIMP)-2 in gingival fibroblasts, as detected by western blotting. The values represent the mean difference in optical density of the band between the Substance P-treated cells and the untreated cells (Substance P at 10^{-9} or 10^{-4} M).

effect on MMP-1, -3, -11 and TIMP-2, which were up-regulated 18-, 4-, 24- and 8-fold, respectively. Less up-regulation was found at the lower Substance P concentration. However, MMP-2 was up-regulated to a similar extent at both Substance P concentrations. Kwong *et al.* also found a weak correlation between gene-expression and protein-expression profiles (25). The amount of protein product can be influenced by post-transcriptional regulatory mechanisms (25), which may explain the present findings. Furthermore, the greater magnitude of up-regulation for mRNA relative to protein may be related to the accumulation of the RNA without degradation.

The extracellular matrix of periodontal tissues consists mainly of collagens: type I collagen is the principal component, in addition to types III, IV, V and VI. Noncollagen proteins, such as elastin, fibronectin, laminin and proteoglycans, are also present in the extracellular matrix (26). These matrix constituents can be degraded by MMP-1, -2, -3 and -11 (19) which, according to our data, are up-regulated by Substance P. However, the increased expression does not necessarily mean increased degradation of matrix constituents, as TIMP-2 levels also increased.

There are other mechanisms through which Substance P plays a role in inflammatory diseases. Substance P exhibits powerful pro-inflammatory properties, causing vasodilatation directly by acting on smooth-muscle cells and indirectly by stimulating histamine release from mast cells (27), increasing microvascular permeability and plasma protein extravasation (28) and inducing interelukin-6 synthesis in monocytes *in vitro* when lipopolysaccharide is present (29).

Bartold *et al.* noted the biphasic nature of Substance P, which slightly inhibits gingival fibroblast proliferation at high concentrations and is stimulatory at low concentrations (17). The present data corroborate the findings of Bartold *et al.* concerning Mantyh's hypothesis (30), according to which Substance P initially promotes and directs the inflammatory and immune response to produce a hyper-inflammatory state, the main outcome of which is destruction of the surrounding tissues. During this early stage, Substance P may be present locally at high concentrations because antidromic stimulation of Substance P immunoreactive fibers will cause the release of large amounts of Substance P into the surrounding tissue. The subsequently increased higher up-regulation of MMP-1, -3 and -11, stimulated by the higher Substance P concentration reported here, may then lead to periodontal destruction. Excessive production of MMPs is associated with periodontitis (13,31–33). After the infection and damaged tissue have cleared, Substance P may continue to be released and function in tissue remodeling, stimulating the reduced lower up-regulation of MMP-1, -3 and -11, which are important in this process.

In our study, MMP-1, -2, -3 and -11 and TIMP-2 expression were markedly affected by treatment with Substance P, revealing their clear regulation by Substance P. However, the expression of TIMP-1 was not affected, suggesting that other factors, such as growth factors and cytokines, may be involved in the regulation of expression. The regulation of TIMP-1 seems to differ from that of the MMPs, particularly in terms of induction by cytokines in gingival fibroblasts (34). In human synovial fibroblasts, Hecker-Kia *et al.* have shown that Substance P stimulation significantly enhanced secretion of MMP-2, whereas no significant increase of MMP-1 and -3 secretions was observed (35).

Fibroblasts apparently express MMP-2 constitutively and express MMP-1, -3, -7 and -11 when induced by growth factors and cytokines (36,37); however, we showed that MMP-1, -2, -3 and -11 are constitutively expressed by gingival fibroblasts. Apparently, such low protein expression was undetected in the immunohistochemical technique employed by Uitto *et al.* (37), explaining our contrasting results. Furthermore, in our study, MMP-7 was not detected by western blotting but was detected by real-time PCR. Real-time PCR may be a more sensitive technique, or post-transcriptional regulatory mechanisms may interfere with subsequent protein translation (25).

In conclusion, in gingival fibroblast cells, Substance P at a concentration of 10^{-4} M induces greater up-regulation of MMP-1, -3 and -11 and TIMP-2 expression, whereas Substance P at a concentration of 10^{-9} M induces diminished up-regulation, which may provide a mechanism for modulating periodontal breakdown.

Acknowledgements

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