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Gene regulation of MMPs and TIMPs by somatostatin in human fibroblasts

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Abstract

Introduction: Somatostatin (SST) is a commonly found neuropeptide with general inhibitory functions. The aim of this study is to evaluate the effect of somatostatin in different concentrations on the mRNA expression of MMPs and TIMPs in cultured, human, gingival fibroblasts. Methodology and resources: Human gingival fibroblasts were stimulated with 10^{-4}, 10^{-9} or 10^{-12}M somatostatin DMEM without fetal calf serum; while untreated fibroblasts served as controls. After the incubation period, the RNA was extracted and the first-strand cDNA was synthesized. Alterations in the expression of MMP-1, MMP-2, MMP-3, MMP-7, MMP-11, TIMP-1 and TIMP-2 mRNA were evaluated using real-time polymerase chain reaction (PCR). β-actin mRNA expression was used to normalize the data. Results: After 24 hours of treatment and at the highest concentration, SSTinduced a down-regulation of MMP-1, -2, -3 and -7 expression, and an up-regulation of MMP-11 expression; while at the lowest concentration the substance induced an up-regulation of MMP-1, -2, -3, TIMP-1 and -2 expression. Similar effects were observed after 72 hours of treatment, except for the up-regulation of TIMP-2 at the higher SST concentration, as well as an up-regulation on MMP-7 and -11 expression and a down-regulation of MMP-2 and TIMP-2 expression at the lower SST concentration. Discussion: The modulation of inflammation by SST is still unclear. The findings of this study suggest that SST can modulate the gene expression of MMPs and TIMPs by cultured fibroblasts and that its effects depend on the concentration. This may represent one of the mechanisms of inflammation suppression by SST.

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Introduction

Homeostasis in the organism is regulated by three interlinked structures: the endocrine, nervous and immune systems.[1] It is increasingly clear that exchanges of information among these

systems are facilitated by the endocrine and/or paracrine release of hormones, neuromodulators and cytokines by any of these systems and by the shared expression of reciprocal receptors for these mediators. Neuropeptides are released from the unmyelinated nerve endings within the central lymphoid organs and peripheral tissues. In addition to their role in endocrine tissues, neuropeptides also play a regulatory role in the human immune system.[2] At the same time, neural cells function as receptors for cytokines, which are released from the immune system in a paracrine fashion and affect neural growth and differentiation.[3] In addition, immune cells can themselves produce neuropeptides, which influence nervous or immune cells in a paracrine or autocrine fashion.[3]

Somatostatin (SST) is a commonly found neuropeptide with a general inhibitory function on hormone release in the anterior pituitary and the gastrointestinal system4. SST also works as a neurotransmitter, immunomodulator, and suppressor of angiogenesis and cell proliferation.[5,6]

SST is released from the capsaicin-sensitive sensory nerve terminals. In the peripheral nervous system, it is found in sympathetic and sensory neurons innervating the lymphoid organs[7]. SST is also found in lymphocytes, macrophages and thymic dendritic cells, as well as in dendritic-type cells near or within the epithelium and single nerve fibers close to the epithelium.[8]

In inflammations, SST shows an anti-inflammatory behavior, generally inhibiting immunoglobulin synthesis, T cell proliferation and splenocyte proliferation[6,9]. In addition, it suppresses the inflammation system by inhibiting the activity of activated dendritic cells on prime T cells[10] and by inhibiting the release of IFN-γ from human T lymphocytes.[11]

Many chronic immune-mediated diseases in which SST is up-regulated, including system lupus erythematosus, rheumatoid arthritis and periodontitis,[12-14] have an important participation of matrix metalloproteinases (MMPs) in their pathogenesis.[15,16]

Usually, MMPs participate in numerous tissue-remodeling processes, in which they are responsible for extracellular matrix degradation. They are involved in physiological processes, such as embryonic development, postpartum involution of the uterus, bone and growth plate remodeling, ovulation, and healing of wounds[17]. Their activity is controlled by the action of tissue inhibitors of matrix metalloproteinases (TIMPs), and an imbalance between levels of MMPs and TIMPs can result in high tissue loss.[17,18]

In periodontitis, MMP regulation seems to occur through 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, epidermal growth factor and substance P *in vitro*.[20-22] However, the impact of SST on the gene expressions of MMPs and TIMPs has not been previously evaluated.

Therefore, we hypothesized that SST can modulate the inflammation response by acting directly on fibroblasts, regulating the expression of MMPs and TIMPs. Thus, the aim of the present study was to investigate whether somatostatin at different concentrations can regulate the expression of the mRNA for MMP-1, -2, -3, -7 and -11, and TIMP-1 and -2 in cultured human gingival fibroblasts.

Methodology and resources

Cell culture in monolayer

The current protocol was approved by the local research Ethics Committee under the number 203/01, and the research was ethically conducted in accordance with the Helsinki Declaration (World Medical Association).

Human gingival fibroblasts obtained from non-smoking, periodontally and systemically healthy gingival explants, were used at the fifth passage[21,22]. Explants were rinsed for 30 seconds 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 1–2mm^3, rinsed three times in phosphate-buffered saline and placed in tissue culture dishes. After 24 hours, a thin layer of Dulbecco's modified Eagle's medium containing 10% antibiotic solution was added to the dishes and the medium was supplemented. Within 14–20 days, 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 then subcultured. For the experiments, fibroblasts were plated in 60mm diameter dishes at 3 x 10^5 cells/ml. 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 24h in Dulbecco's modified Eagle's medium without fetal calf serum. Cell cultures were then exposed to somatostatin (Sigma) at 10^{-4}, 10^{-9} or 10^{-12} M for 24 and 72 hours in DMEM without fetal bovine serum. Untreated cells served as controls. All experiments were performed in triplicate.

Sample preparation

After incubation, 100-µL aliquots of fibroblast culture medium were collected. Ten microlitres of protease inhibitor cocktail (Sigma) were added and the aliquots were stored at -70°C. The fibroblasts were then trypsinized, resuspended in 100µL of phosphate buffered saline, lysed by adding 0.9mL of TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) and their RNA was 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 doublestranded 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, 1µg of RNA sample, 1µL of random primer (Random Primers; Invitrogen), 1µL of 10mM dNTP Mix (Invitrogen) and distilled water to 13µL were added to a microcentrifuge tube, heated to 65°C for 5 minutes and chilled on ice. Then, 4µL of 5x First-Strand Buffer and 2µL of 0.1M dithiothreitol were added and incubated at 37°C for 20 seconds. One microlitre of Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen) was added and the tubes were incubated at 37°C for 50 minutes.

Real-time PCR

Alterations in the expression of mRNA for the MMP-1, MMP-2, MMP-3, MMP-7, MMP-11, TIMP-1 and TIMP-2 genes were evaluated using real-time PCR (7300 Real Time PCR System, Applied Biosystems, Foster City, CA, USA) and SYBR Green PCR Master Mix (Applied Biosyste-

ms). β-actin mRNA expression was used as a control to normalize the gene expression data. All real-time PCR assays were performed in 96-well optical plates (Applied Biosystems) using the following cycling parameters: 50°C x 2 minutes and 95°C x 10 minutes; PCR cycling for 40 cycles at 95°C x 15 seconds (denaturation), 60°C x 1 minute (annealing) and a dissociation cycle (95°C x 15 seconds, 60°C x 30 seconds, and 95°C x 15 seconds).

Oligonucleotide primers for MMP-1, -2, -3, -7, -11 and β-actin were designed from sequences in the GenBank database using Custom Primers-OligoPerfect™ Designer (Invitrogen). One sample of PCR product obtained with each set of primers was fully sequenced (MegaBace, Amersham Bioscience Corp, CA, USA). The sequencing reactions were prepared using DYEnamic™ ET Dye Terminator Cycle Sequencing Kit for MegaBACE™ DNA Analysis Systems (Amersham Bioscience Corp) in accordance with the manufacturer's protocol. The samples were precipitated using Isopropanol, resuspended in MegaBACE loading solution and sequenced. The primer sequences were deposited in GenBank under appropriate accession numbers, as previously described.[21,22] Primer sequences for TIMP-1 and -2 were obtained from the literature.[23,24] 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) C_T 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 somatostatin-treated cells and the control cells was computed ($\Delta\Delta C_T = \Delta C_T$ for somatostatin-treated cells minus ΔC_T for control cells). The range of gene expression for each somatostatin concentration was then estimated from the relation $2_T^{\Delta\Delta C}$.

Data analysis

The collected data from all groups were imported to Statistical Package for Social Sciences (SPSS) for Windows software, version 26.0 (SPSS Inc., Chicago, IL, USA). A Kruskal–Wallis ANOVA, followed by the Mann–Whitney test, was used to evaluate the significance of the differential effect of SST on the different target genes and to test the significance of the effect of different SST concentrations on each target gene expression. Significance level was set at 5%.

Results

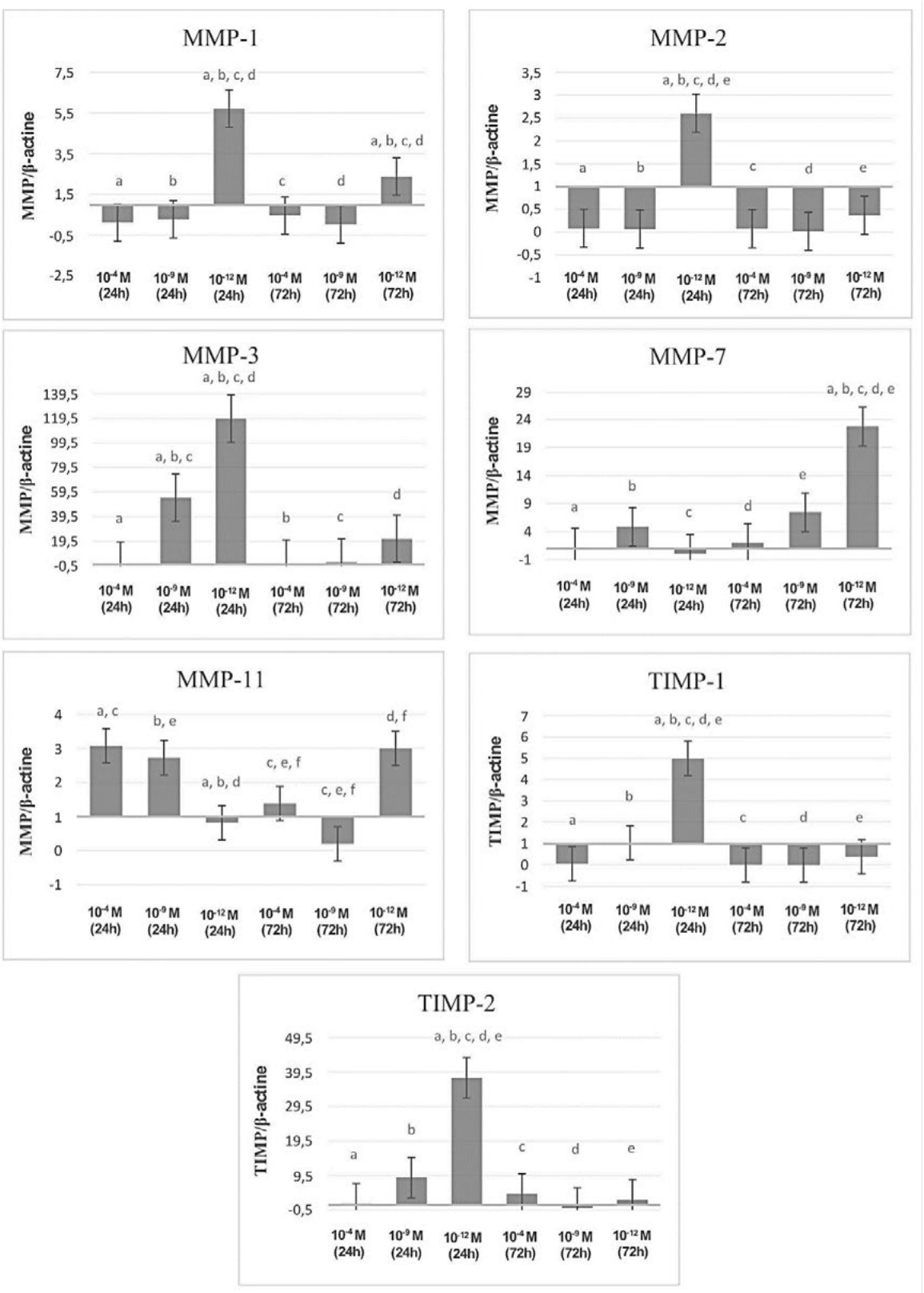
The effect of somatostatin on mRNA expression of MMP-1, MMP-2, MM-3, MMP-7, MMP-11, TIMP-1 and TIMP-2 genes in gingival fibroblasts, is illustrated in Figure 1.

Somatostatin (SST) had no significant effect on β-actin mRNA expression ($p=0.44$), validating the usefulness of the latter as an internal control.

The comparison of different genes showed significant ($p<0.01$) differences in the ratio of up/down-regulation; except for the comparisons between MMP-7 x TIMP-2, MMP-1 x MMP-11 and TIMP-1, MMP-3 x TIMP-2, and MMP-11 x TIMP-1 ($p>0.05$).

At the highest SST concentration and 24-hour exposure, MMP-1 and -2 were down-regulated; however, they were up-regulated at the lowest SST concentration. For 72 hours, SST down-regulated their expression; except for the lowest dose/72-hour exposure, which up-regulated the MMP-1 expression ($p<0.05$) by a factor of 2.

Figure 1. Effect of somatostatin on mRNA expression of MMP-1, MMP-2, MM-3, MMP-7, MMP-11, TIMP-1 and TIMP-2 genes in gingival fibroblasts as detected by semi-quantitative, real-time RT-PCR



Legend: The values (mean±SD) represent the gene expression range for each somatostatin concentration (10^{-12}, 10^{-9}, 10^{-4}M) in 24 and 72 hours, estimated using the expression: $2^{-\Delta\Delta C}_{T}$.Values greater than 1 indicate up-regulation, and values smaller than 1 indicate down-regulation. For values followed by the same letter, the difference was statistically significant.

MMP-3 was down-regulated at the highest SST concentration and up-regulated at the lowest concentration ($p<0.05$). MMP-7 was highly up-regulated at the lower SST concentration in 72-hour treatment; otherwise, MMP-7 was down-regulated or less up-regulated at higher SST concentrations ($p<0.05$). At 24-hour treatment, MMP-11 was up-regulated at higher SST concentrations and down-regulated at the lowest concentration. On the other hand, at the lowest SST concentration, MMP-11 was up-regulated when the exposure time was increased to 72 hours ($p<0.05$).

TIMP-1 was up-regulated at the lowest SST concentration at 24 hours of treatment, and longer exposure resulted in a down-regulation ($p<0.05$). TIMP-2 was highly up-regulated at lower SST concentrations at 24h; however, at 72 hours it was highly up-regulated at the highest SST concentration ($p<0.05$).

Discussion

Our results showed that 24-hour exposure of gingival fibroblasts to SST at a high concentration (10^{-4} M) induced a down-regulation of MMP-1, -2, -3 and -7 expression, and an up-regulation of MMP-11 expression; while at a low concentration (10^{-12}M) the exposure induced an up-regulation of MMP-1, -2, -3, TIMP-1 and -2 expression. Seventy-two hours of treatment still resulted in an up-regulation on TIMP-2 expression at the highest SST concentration, as well as an up-regulation of MMP-7 and -11 expression and a down-regulation of MMP-2 and TIMP-2 expression at the lowest SST concentration. Therefore, the functions of neuronal SST in inflammation are related to a variety of mechanisms, including the effect on immune cells,[9-11] the inhibitory effect on pro-inflammatory neuropeptide release[25] and, as our results suggest, the effect on MMP and TIMP expression in fibroblasts.

The low concentrations of SST in this study are similar to those described during inflammation. The serum concentration of SST in patients with rheumatoid arthritis aged 55 years or older is 1.5×10^{-11}M[26] and the intravitreal levels of SST in diabetics is 4.5×10^{-11}M.[27] Physiological SST concentrations are heterogenous in the organism and the SST concentration of the periodontium was not measured, although our results indicate that it is above 10^{-12}M and below 10^{-9}M.

The anti-inflammatory effect of SST affects many tissues. SST suppresses the inflammatory effect in cutaneous lymphocytic inflammatory and tumoral infiltrates.[28] In human retinal pericytes, SST can neutralize the effects of pro-inflammatory factors, up-regulate pro-apoptotic mediators and down-regulate pro-survival factors, mediated by microglia.[29] In acute pancreatitis, an increase in SST concentrations occurs, which is correlated with interleukin-6 levels.[30] In pancreatic fistulas in rats, the efficacy of the SST analogue in reducing inflammation is higher than that of other drugs, with greater reduction in interleukin-6 and tumor necrosis factor-α concentrations.[31] Systemic or intra-articular injection of SST prompted a reduction of joint inflammation in experimental animals with CFA-induced arthritis.[32] Chronic arthritis in rats is accompanied by the release of SST into the circulatory system, which, in turn, leads to diminished inflammatory response by: (a) inhibiting the release of proinflammatory neuropeptides from afferent nerve endings; (b) acting directly on blood vessels by decreasing vasodilation and plasma protein extravasation; and (c) inhibiting immune cell functions.[33]

This anti-inflammatory effect of SST is related, at least in part, to its action on fibroblasts. The way in which process occurs, however, remains unknown. In synovial membranes of patients

with rheumatoid arthritis, the exposure of fibroblasts to octreotide, an SST analogue, results in the inhibition of pro-inflammatory cytokines (interleukin-15 and tumor necrosis factor-α) and increased concentrations of anti-inflammatory cytokine (interleukin-10).[34]

Proinflammatory neuropeptides, such as substance P (SP) and calcitonin gene-related peptide, are up-regulated in primary afferent neurons in acute and chronic inflammation,[35] and SST, which is an antinociceptive and anti-inflammatory neuropeptide, is also regulated in inflammation.[12,13,21]

The presence of neuropeptides was verified in the gingival crevicular fluid and the periimplant sulcus in healthy and diseased gums. Diseased sites presented an increase in pro-inflammatory neuropeptides and a decrease in neuropeptides related to immunosuppressive effects.[36] In periodontitis, SST mRNA is up-regulated in the mandibular division of the ipsilateral trigeminal ganglion[14] and in SST-immunoreactive cells in the gingival of phenytoin-treated patients.[37] The degree of tissue destruction in those inflammatory diseases may be related to the balance of pro- and anti-inflammatory and nociceptive peptide expression, which regulates the expression of MMPs, the main enzyme associated with extracellular matrix degradation.

SP at a high non-physiological concentration induces a high up-regulation of MMP-1, 2, 3 and 11 and of TIMP-1 and 2 expressions by gingival fibroblasts,[22] which can result in tissue breakdown. Furthermore, according to the present study, SST at high concentration, in general, induces down-relation of these genes, which can inhibit tissue breakdown. The controversial findings for the different MMPs expressions in this study may be related to their functions. MMP-11 has low destructive potential and is incapable of degrading proteins with major relevance in the extracellular matrix,[38] while MMP-7 shows similar levels in crevicular fluid to patients with periodontal diseases in comparison with healthy patients, indicating a role in innate host defense of periodontal tissues.[39]

Our results may clarify how SST can modulate the inflammatory process. However, this is an *in vitro* study, and its findings may not represent how this process actually occurs *in vivo*. New research on cell viability using these concentrations of SST should be performed, in an attempt to ensure that they are not toxic. Furthermore, the lack of western blotting to confirm real-time PCR findings is another limitation.

Conclusion

Somatostatin can modulate the expression of MMPs and TIMPs in cultured gingival fibroblasts. In general, at high concentrations SST down-regulates MMP expression and up-regulates TIMP-1 expression,. This may represent one of the mechanisms of inflammation suppression by somatostatin.

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