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Effect of Substance-P on Metalloproteinase Release and Extracellular Matrix Accumulation of Articular Chondrocytes

Ryuichi Saura¹, Soichiro Hirata¹, Hitoshi Ishikawa¹, Kosaku Mizuno²

Clinical evidences have suggested that the peripheral nervous system might be involved in the pathophysiology of inflammation. The neuropeptide substance P (SP), which has been found in unmyelinated C fiber of the peripheral sensory nerve, is reported to be one of the possible mediators of chronic inflammation, since it has been detected in inflamed joints and has various biological effects on mesenchymal cells. It is also reported that SP induced collagenase release of synoviocytes obtained from patients with rheumatoid arthritis. However, whether or not SP induces articular cartilage degradation directly, remains unknown. Therefore we have investigated the effect of SP on matrix metalloproteinase release and the accumulation of sulfated glycosaminoglycan of the articular chondrocyte in vitro.

Administration of exogenous SP resulted in the increase of metalloproteinase release in the monolayer cultured bovine articular chondrocytes. Significant increase (p<0.05) was observed at the concentration of 10⁻⁹ M for 48 h incubation. Meanwhile SP had no effect on sulfated glycosaminoglycan synthesis of the articular chondrocyte. These results suggest that SP may increase the deterioration of articular cartilage through augmentation of the release of metalloproteinase in inflammatory arthritis.

Keywords
Articular chondrocyte, Substance-P, Collagenase, Stromelysin, Matrix metalloproteinase, Cartilage matrix degradation.

INTRODUCTION

In rheumatoid arthritis (RA) with hemiplegia, the joint in paralyzed side is spared from the inflammatory process.¹ ² This clinical observation is supported by reports, stating that joints developing severe arthritis are more densely innervated by substance-P (SP), contained in primary afferent neurons, and that infusion of SP into the joints increases the severity of adjuvant-induced arthritis in rats.³ Moreover, SP was detected in rheumatoid joints⁴,⁵ and had various biological effects on the mesenchymal cells, including synoviocytes⁶ and monocytes.⁷ Neuropeptide SP, therefore, seems to be one of the mediators of neurogenic inflammation.⁸ In order to investigate the interaction of SP with articular chondrocytes, therefore, we have studied the biological effects of this neuropeptide on the metabolism of cultured articular chondrocyte in
MATERIALS AND METHODS

1. Chondrocyte culture.

Articular cartilage was shaved from bovine carpo-metatarsal joints, diced, and digested for 6 hours at 37 °C in Dulbecco’s modified Eagle's medium (DMEM; Gibco BRL., Grand Island, NY, USA) containing 0.1 % collagenase (Washington Biochemical Corp., Freehold, NJ, USA). The articular chondrocytes were harvested by filtration through gauze to remove tissue fragments, followed by centrifugation (2000 rpm for 10 min.). The cells were washed 3 times and resuspended in DMEM, supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Biowhittaker, Walkersville, ML, USA) and antibiotics (20 units/ml penicillin and 100 μg/ml streptomycin). This complete medium was used in all chondrocyte assays unless stated otherwise. The isolated cells, which were routinely checked for rounded (cobble stone) morphology, consistent with the differentiated phenotype⁹,¹⁰, were cultured in a monolayer fashion, then subjected to experiments described below.


In order to examine the effect of SP (Sigma Chemical Co., St. Louis, MO, USA) on stromelysin release of the articular chondrocyte, a caseinolytic activity in the culture media was measured by ¹⁴C-labeled casein (Sigma) digestion as described previously with some modification.¹¹ Articular chondrocytes were allowed to attach in every well of 24-multiwell flat bottomed culture plates (Corning Glass Works, Corning, NY, USA) at the cell density of 2.5 x 10⁵ cells/well and cultured for 24 hours in the complete medium. On the following day, after gently being washed with 0.05 M phosphate buffered saline (pH 7.4), the medium was changed to DMEM with 2% FBS. After the chondrocytes were cultured in the presence of various concentrations of SP for 72 hours, the sample aliquots were assayed as follows. Reaction mixtures contained 20μl of labeled casein, 100μl of assay buffer containing 1 mM amino phenylmercuric acid (APMA; Sigma) to activate the latent form of the stromelysin and sample aliquots to give a total volume of 220μl. Reactions were performed for 4 hours at 37°C and then terminated by adding 100μl of "cold" casein and 150μl of 10 % trichloracetic acid (TCA; Sigma). The mixture was centrifuged and the enzyme activity determined as the release of TCA soluble radioactivity in the supernatant by scintillation counter (Packard Instrument Company, Meriden, CT, USA). One unit of casein degrading activity is defined as the degradation of 1μg casein/min. at 37°C.


In order to examine the effect of SP on collagenase release of articular chondrocyte, a collagenolytic activity was determined in the culture media by using FITC-labeled collagen as substrate. Assay procedure for col-
lagenolytic activity was performed according to the manufacturer's instructions (Collagen Gijutsu Ken-syuikai, Tokyo, Japan). Briefly, articular chondrocytes were cultured for previous 24 hours in the complete medium in 24-multiwell culture plates and stimulated with various concentrations of SP for the following 72 hours in DMEM with 2% FBS. The sample aliquots were assayed. Two hundred µl of 0.05% FITC-labeled collagen in Tris/HCl (pH 7.5) were mixed with the sample aliquots containing 1 mM APMA to activate the latent form of the collagenase to give a total volume of 400 µl. Reactions were performed for 3 hours at 35°C and then terminated by adding 10 µl of “stop solution” (80 mM o-phenanthroborin). The digested fragment was extracted by adding 400 µl of 70% ethyl alcohol and, after centrifugation (3000 rpm for 10 min.), fluorescent intensity in the supernatants was determined by spectrofluorometer (Japan Spectroscopic Co., LTD., Tokyo, Japan). One unit of collagen degrading activity is defined as the degradation of 1 µg collagen/min. at 37°C.


The glycosaminoglycans (GAG) accumulation in both the cell layer and the culture media was measured by incorporation of [35S]O4²⁻ (New England Nuclear, Boston, MA, USA) into the newly synthesized extracellular matrix of the articular chondrocyte as described previously with some modification. Two hundred fifty thousand articular chondrocytes were inoculated in every well of 24-multiwell flat bottomed culture plates and cultured for 24 hours in complete medium. The following day, the medium was changed to 2% FBS-DMEM supplemented with 148 KBq of [35S]O4²⁻. After the chondrocytes were cultured in the presence of various concentrations of SP for 72 hours, the culture medium was collected. Then the cell layers were solubilized by 300 µl of 0.15M NaOH and neutralized by adding 0.15 µl of 3 M HCl. Both cell lysate and collected culture media were digested by 8 µg of pronase E (Kaken Seiyaku Co. Tokyo, Japan) for 12 hours at 55°C. After digestion, GAG were aggregated by adding 1% cetylpyridinium chloride (Wako Pure Chemical Industries LTD., Osaka, Japan) and gathered on the filter membrane (Milipore Corporation, Bedford, MA, USA). The radio-activity on the membrane was determined by scintillation counter (PACKARD INSTRUMENT COMPANY, Mariden, CT, USA).

5. Statistical analysis.

Statistical analysis were carried out on all data points with regard to control by a Student's t-test. Each data point represented the mean of four separate samples with the corresponding standard error of the mean (SEM). P values under 0.05 were considered significant, statistically seen.

RESULTS

1. Effect of SP on matrix metalloprotease release from cultured articular chondrocyte.
The effect of SP on stromelysin release of the articular chondrocyte was detected as a caseinolytic activity in the chondrocyte culture media. Articular chondrocytes were cultured in the presence of various concentrations of SP in vitro. These cells in DMEM supplemented with 2% FBS released the detectable amounts of caseinolytic activity without stimulation for 72-hour incubation. The release of this caseinolytic activity from articular chondrocytes was enhanced by increasing the concentration of SP (Fig.1). Significant increasing was observed at over $10^{-9}$ M of SP ($P<0.05$). This concentration is reported to be detectable in the inflamed joint such as rheumatoid arthritis (RA).

This stimulating effect of SP was dependent on the incubation time and a significant increase of the caseinolytic activity was attained over 48-hour incubation when chondrocytes were treated with $10^{-8}$ M of SP (Fig.2).

In terms of the release of collagenolytic activities from articular chondrocytes, SP also enhanced to release the collagenolytic activities in a dose-dependent manner. Significant increase was observed when articular chondrocytes were incubated with over $10^{-8}$ M of SP for 72 hours (Fig.3).

2. Effect of SP on accumulation of the extracellular matrix around the cultured articular chondrocyte.

Articular cartilage is composed of both chondrocytes and the extracellular matrix. Downregulation of the extracellular matrix synthesis of chondrocytes by stimulation of

![Figure 1. Effect of SP on stromelysin release from cultured articular chondrocyte.](image)

**Fig. 1.** Effect of SP on stromelysin release from cultured articular chondrocyte.

The effect of SP on stromelysin released from the articular chondrocyte was detected as described in materials and methods. Articular chondrocytes cultured in the absence of SP released detectable amounts of caseinolytic activity for 72-hour incubation. The release of this caseinolytic activity from the articular chondrocytes was enhanced by increasing concentration of SP. Significant increase was observed at over $10^{-9}$ M of SP ($P<0.05$).

![Figure 2. Time kinetic study of the SP-induced matrix metalloproteinase release from cultured articular chondrocyte.](image)

**Fig. 2.** Time kinetic study of the SP-induced matrix metalloproteinase release from cultured articular chondrocyte.

The time kinetics of SP-induced stromelysin released from the articular chondrocyte was examined. Articular chondrocytes cultured in the presence of $10^{-8}$ of SP released a detectable amount of caseinolytic activity in the incubation time dependent manner. The release of this caseinolytic activity from the articular chondrocytes was significantly increased by SP over 48-hour incubation ($P<0.05$).
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Effect of SP on collagenase release from cultured articular chondrocyte.

The effect of SP on collagenase released from the articular chondrocyte was examined as follows. In order to examine the effect of SP on collagenase released from the articular chondrocyte, collagenolytic activities were determined in the culture media by using FITC-labeled collagen as substrate. Articular chondrocytes cultured in the presence of various concentration of SP released the collagenolytic activities in a dose-dependent manner. Significant increase was observed at the level over $10^{-8}$ M.

**DISCUSSION**

The deterioration of functional property of the cartilage matrix is commonly observed in chronic arthritis such as osteoarthritis and RA. The mechanism involved seems to be a degradation of the extracellular matrix components and an injury of chondrocytes located in the cartilage lacunae. It was reported that the cartilage matrix is degraded by proteolytic enzymes from the inflammatory cells, such as polymorphonuclear leukocytes and macrophages, synovium and chondrocytes.

Various kinds of stimuli are reported to induce the cartilage matrix degradation by activating articular chondrocytes. Interleukin 1, which is one of these mediators and which is highly detected in the arthritic joint, is indicated to play an important role in the cartilage degradation due to stimulation of the release of metalloproteinases, PGE$_2$, and to suppress the GAG synthesis.
The neuropeptide substance P, which has been found in the unmyelinated peripheral sensory nerve, is also reported to be one of the possible mediators of chronic inflammation. There is evidence that SP-induced neurogenic inflammation contributes to the pathophysiology of chronic arthritis. Substance P may play an important role in inflammatory process through activation of a variety of inflammatory cells including polymorphonuclear leukocytes, lymphocytes, mast cells, macrophages and monocytes.

We have studied the effect of the SP on articular chondrocyte function in this paper. Substance P has induced both stromelysin and collagenase release from the articular chondrocyte at the low concentration of SP (10^{-9}-10^{-6} M) while no effect of SP on the GAG accumulation around the chondrocytes was observed. These proteolytic activities are activated with pretreatment of 1 mM APMA (data not shown), which is suggested that these are members of metalloproteinases and released as the latent forms.

In terms of the modulation of chondrocyte metabolism by SP, it is already reported that neither collagenase release nor PGE2 secretion of articular chondrocyte were enhanced by whole molecule of SP, but induced by C-terminal fragment SP- (7-11). Whole molecule of SP has affected neither proteoglycan nor protein synthesis of articular chondrocyte in vitro. It is also reported that neither N-terminal fragment SP- (1-6) nor other mammalian tachykinins, such as neurokinin A or neurokinin B, stimulated collagenase and PGE2 secretion of chondrocytes. The receptor-mediated activation is reported to require a low concentration (10^{-9}-10^{-6} M) of SP. Whereas, a high concentration of SP is required to release histamine from rat peritoneal mast cell through N-terminal domain sequence of SP in vitro. It is postulated that SP can directly activate G proteins independent of a specific SP receptor. The previous report indicated the possibility that a high concentration of C-terminal fragment SP- (7-11) may directly activate the \( \alpha \) subunit of G protein, resulting in stimulation of PGE2 and collagenase synthesis.

The detailed intracellular mechanisms of the induction of metalloproteinase released by a low concentration of whole molecule of SP in articular chondrocytes has not been fully investigated in this study. However the results of the present study are showing that SP induces the release of matrix metalloproteinase, such as stromelysin and collagenase in the articular chondrocyte directly. In conclusion, substance-P may be one of the important mediators involved in the mechanism of the cartilage degradation in chronic arthritis.
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