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Participation of substance P distribution in the cytokine production of rheumatoid synovium

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Abstract Based on findings which suggested the involvement of the neuropeptide substance P in the pathogenesis of rheumatoid arthritis (RA), we investigated the mechanism of synovial pannus formation in RA, and examined the interaction between the cytokine production of synovial tissues and the concentration of substance P in the cartilage–pannus junction (CPJ). The CPJ and other peripheral synovial tissues were separately obtained from each part of the synovium from the knee joints of seven RA patients. The concentrations of substance P and the cytokines interleukin (IL)-1β and IL-6 in the CPJ and peripheral synovial tissues were determined by enzyme-linked immunosorbent assays. In addition, synovial cells were isolated from the CPJ and peripheral synovial tissues and treated with substance P or neurokinin-1 receptor antagonist to analyze the changes in cytokine production. The substance P levels were 211.2 and 50.5 pg/mg protein in the CPJ and the peripheral synovium, respectively. The IL-1β and IL-6 levels in the CPJ were 24.6 and 12.8 pg/mg protein, respectively. In the peripheral synovium, these levels were 4.3 and 2.5 pg/mg protein, respectively. In the CPJ, the IL-1β and IL-6 levels in tissue containing a high concentration of substance P (>200 pg/mg protein) were 39.4 and 21.6 pg/mg protein, respectively, and those in tissue containing a low concentration of substance P (≤200 pg/mg protein) were 11.6 and 5.1 pg/mg protein, respectively. Synovial cells from the CPJ produced higher levels of IL-1β and IL-6 than those from peripheral tissues. In addition, treatment of the cells with an NK-1 antagonist significantly reduced the production of these cytokines by the synovial cells. The theory that substance P plays a role in the pathogenesis of RA via the upregulation of cytokine production should be considered in further studies on the immunomodulatory properties of substance P in arthritis.

Key words Cytokine production · Substance P · Synovial cells · Rheumatoid arthritis

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Introduction

Recently, an increasing number of reports have been published relating to evidence supporting the involvement of the nervous system, and in particular the neuropeptide substance P, in the pathogenesis of rheumatoid arthritis (RA). In an adjuvant arthritis models in rats, the depletion of endogeneous substance P ameliorated the conditions caused by the disease, while the administration of substance P aggravated those conditions.[1] In addition, increases in the concentration of substance P in the synovial fluid of RA patients, as well as in the blood plasma, have been reported.[2]

Substance P has been described as an immunostimulatory neuropeptide that can modulate the function of several cells in humans, including T and B lymphocytes, monocyte/macrophage cells, polymorphonuclear leukocytes, and synovial cells.[3] Since many findings have confirmed the role of synovial cells in the pathogenesis of RA, an investigation of the inflammatory effects of substance P on synovial cells in RA patients is important as a model for analyzing possible alterations in the interaction between the immune and nervous systems in RA.[4] However, only a limited amount of information is available on the effects of substance P on the synovium in RA. We examined the effect of substance P on synovial cells obtained from different sites of synovial tissue to clarify the mechanism of pannus formation in the cartilage–pannus junction (CPJ).[5,6]

The aim of this study was to investigate the effects of substance P on the production of inflammatory cytokines such as interleukin (IL)-1β and IL-6 in a series of RA patients.

Patients and methods

Patients

The subjects were seven patients (five females and two males, mean age 50 years, range 31–67 years) with RA as defined by the revised criteria published by the American Rheumatism Association. The median duration of the disease was 5 years (range 2–25 years). Erosions were detected in four patients, and nodules in two. Tests for rheumatoid factor were positive in all patients. All patients were taking medication; seven were taking nonsteroidal anti-inflammatory drugs (NSAIDs), four were taking prednisolone (5.0 mg/day), and five were receiving "disease-modifying" drugs (gold, bucillamine).

Materials

Substance P was obtained from the Peptide Institute (Osaka, Japan). Phorbol 12-myristate 13-acetate (PMA), calcium ionophor (A23187), β-mercaptoethanol, leupeptine, aprotinin, phenylmethylelsulfonyl fluoride (PMSF), and adenosine 5′-triphosphate (ATP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethylene glycol bis(β-aminoethyl-ether)-N,N,N′,N′,-tetraacetic acid (EGTA) and ethylenediamine-N,N,N′,N′,-tetraacetic acid (EDTA) were obtained from Nacalai Tesque (Kyoto, Japan). Collagenase (type II) was obtained from Worthington Biochemical Co. (NJ, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and amphotericin B were obtained from ICN Pharmaceuticals (CA, USA).

Preparation of synovial tissue samples for assays

Synovial tissue was isolated from the knee joint of each patient during joint replacement surgery, as described below. In this study, the area 3 cm or more from the junction of the synovium and the cartilage was defined as the peripheral synovial tissue. The CPJ was defined as the area at a distance of less than 1 cm from the same junction. Using these these definitions, we gathered synovial tissue during joint replacement surgery. The synovial tissue was maintained in DMEM immediately after collection. The dissected tissue was homogenized in 5 ml phosphate-buffered saline, and centrifuged at $2000 \times g$ for 15 min. The supernatant was filtered through a 0.45-μm filter (Millex-HA, Millipore, Molsheim, France) and centrifuged at $22000 \times g$ for 30 min. The supernatant of each tissue homogenate was maintained at −40°C until the cytokine measurements were performed.

Culture of synovial cells and substance P treatment

Synovial tissue was cut into 2–3 mm^3 pieces and then incubated in Hank's balanced salt solution (HBSS, in mM: 136.8 NaCl; 5 KCl; 1.0 $CaCl_2$; 0.8 $MgSO_4$; 0.3 Na_2HPO_4; 0.4 KH_2PO_4; 20 HEPES; 4.1 $NaHCO_3$; 5.5 glucose; pH 7.4) containing 0.2% collagenase (type II) for 60 min with occasional stirring. The cell suspension was centrifuged for 10 min at $600 \times g$, and the resulting pellets were washed and resuspended in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 units/ml), streptomycin (100 μg/ml), amphotericin B (750 ng/ml), and L-glutamine (2 mM). The cells were then placed on 35-mm microwell dishes (1×10^5 cells/dish), and cultured for 7 days at 37°C in a humidified chamber containing 5% CO_2:95% air. After 1 week of culture without any treatment, synovial cells were treated with substance P. Substance P was then added to the final concentration of 10^{-8} M to 10^{-3} M. After treatment with each concentration of substance P for 1 week, the medium was collected and then immediately frozen and stored until the assays were conducted.

Substance P and cytokine assay

The supernatants of synovial homogenates were lyophilized and used for further analysis. The concentration of substance P was determined by a specific and sensitive (detection limit 1–4 fmol) enzyme-linked immunosorbent assay (ELISA) kit (Neogen, MI, USA). The concentrations of IL-1β and IL-6 in the supernatants of synovial homogenates and culture media of synovial cells were determined by employing a different commercially available ELISA kit (Cistron Biotechnology, Pinebrook, NJ, USA). The sensitivity levels for IL-1β and IL-6 were 4 and 8 pg/ml, respectively.[7]

Neurokinin-1 (NK-1) receptor antagonist treatment

Synovial cells were cultured for 7 days, then pretreated with an NK-1 receptor antagonist, GR82334 (1 μM), in all synovial cells from each part of the synovium before substance P (30 μM) treatment. GR82334 was added 10 min before substance P was added to the medium, and new substance P was used every week.

Statistical analysis

Student's *t*-test was used for statistical analysis. Differences between mean values with P values less than 0.05 were considered significant.

Results

Table 1 shows the concentrations of substance P in the supernatants of the CPJ and peripheral synovium homogenates. Microscopical techniques were employed to collect synovial tissue from two areas located in the innermost layer containing villi, the CPJ and peripheral synovial tissue areas, as described above. The size of each cut was 5 mm × 5 mm, and each of the CPJ and peripheral samples

Table 1. Concentrations (pg/mg protein) of substance P, IL-1β, and IL-6 in the supernatants of the cartilage–synovial junction (CPJ) and peripheral synovium homogenates

	CPJ	Peripheral synovium
Substance P	211.2 ± 30.1**	50.5 ± 23.4
IL-1β	24.6 ± 9.3**	4.3 ± 2.1
IL-6	12.8 ± 8.3**	2.5 ± 1.2

Cells were obtained from the CPJ and the peripheral synovium of 7 RA patients. The substance P, IL-1β, and IL-6 concentrations were measured by ELISA kits as described in the Patients and Methods section. Data represent the mean ± SE of 21 sample preparations from 7 patients

** $P < 0.01$ compared with epipheral synovium

Table 2. Concentrations (pg/mg protein) of IL-1β and IL-6 in the supernatants of the CPJ synovium homogenates containing a substance P level over, less than, or equal to 200 pg/mg protein

	SP > 200	SP ≦ 200
IL-1β	39.4 ± 12.5**	11.6 ± 6.3
IL-6	21.6 ± 9.7**	5.1 ± 3.5

The IL-1β and IL-6 concentrations were measured by ELISA kits. Data represent the mean ± SE of 15 sample preparations from 5 high-substance-P patients and 6 sample preparations from 2 low-substance-P patients

** $P < 0.01$ compared with SP ⩽ 200

were taken in triplicate from each patient from three separate locations. The 21 samples were homogenized in 1 ml phosphate-buffered saline, and the cytokines they contained were found for each unit of protein in 0.5 ml supernatant.

The CPJ synovium contained significantly more substance P than the peripheral synovium. The substance P level in the CPJ was 211.2 ± 30.1, and that in the peripheral synovial tissue was 50.5 ± 23.4 (pg/mg protein). The IL-1β and IL-6 levels in the CPJ tissue were 24.6 ± 9.3 and 12.8 ± 8.3 pg/mg protein, respectively, and those in the peripheral synovial tissue were 4.3 ± 2.1 and 2.5 ± 1.2 pg/mg protein, respectively.

As mentioned above, a comparison between the CPJ and peripheral areas showed that the amounts of substance P contained in the tissue from these areas was clearly different. Therefore, we examined whether or not the differences between the CPJ and peripheral synovial tissues in the production of IL-1β and IL-6 were dependent on substance P. If these differences were dependent on the existence of substance P, a significant difference in the production of IL-1β and IL-6 would also be observed between areas with high and low levels of substance P in the CPJ. A sample was delimited with 200 pg/ml, which was the average substance P content in the CPJ, and we determined whether or not a significant difference in IL-1β and IL-6 production existed between concentrations over 200 and under 200 pg/mg protein. In the CPJ synovium, the IL-1β and IL-6 levels in the tissue containing a high concentration of substance P (>200 pg/mg protein) were 39.4 ± 12.5 and 21.6 ± 9.7 pg/mg protein, respectively, and those in tissues containing a low concentration of substance P (⩽200 pg/mg protein) were 11.6 ± 6.3 and 5.1 ± 3.5 pg/mg protein, respectively (Table 2). Therefore, the IL-1β and IL-6 levels in patients with a high substance P level were considerably greater than those in patients with a low substance P level. However, the result of the analysis of this correlation was not definitive owing to the limited number of cases in the present study.

We then used cultured synovial cells to determine whether the changes in the cytokine concentration was due to direct action by the substance P contained in the tissue. No differences were demonstrated in the cultured CPJ or peripheral synovial tissue cells before the addition of substance P. The measurement of IL-1β production revealed

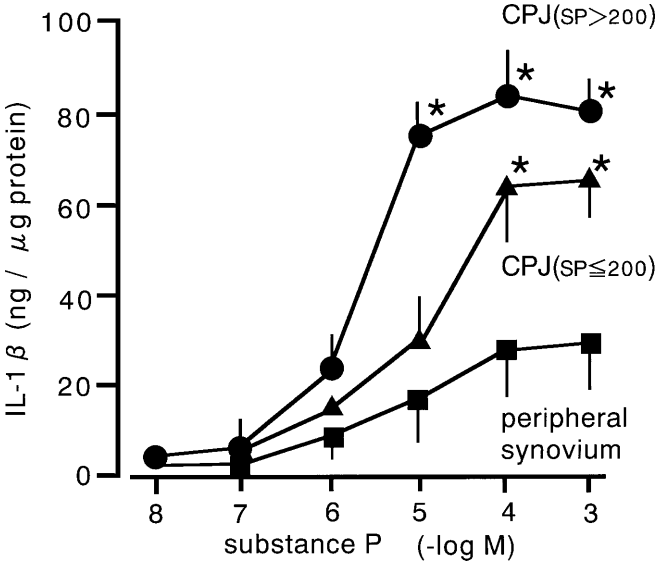

Fig. 1. Dose–response curves of substance P-induced IL-1β production by cultured synovial cells from the cartilage–pannus junction (CPJ) and peripheral synovium. The IL-1β production was assessed as described in the text. Each symbol represents the mean ± SE of 30 preparations. * $P < 0.01$ compared with the peripheral synovium

that substance P (at concentrations ranging from 10^{-8} M to 10^{-3} M) dose-dependently induced IL-1β production by cultured synovial cells obtained from both the CPJ and peripheral synovium (Fig. 1). In addition, cells from the CPJ synovial tissues showed a significantly higher IL-1β production compared with the peripheral synovial cells. Substance P-stimulated IL-1β production was more remarkable in cells from the CPJ tissue containing a high level of substance P (⩽200 pg/mg protein) than that observed in tissue showing a low substance P-stimulated level (>200 pg/mg protein) (Fig. 1).

Measurements of IL-6 production showed that substance P (at concentrations ranging from 10^{-8} M to 10^{-3} M) dose-dependently induced IL-6 production by synovial cells from both the CPJ and peripheral synovium, and that CPJ synovial cells containing a high concentration of substance P produced significantly more IL-6 compared with cells from the peripheral synovium, at a concentration of 10^{-5} M ($P <$ 0.01). The substance P-stimulated IL-6 production results were essentially the same as those for the IL-1β production (Fig. 2).

We then used an NK-1 receptor antagonist to confirm the stimulatory effect of substance P on IL-1β and IL-6 production by the synovial cells. Both the IL-1β and the IL-6 production induced by substance P (30μM) were significantly inhibited by the NK-1 receptor antagonist GR82334 (1μM) in all synovial cells from each part of the synovium (Fig. 3).

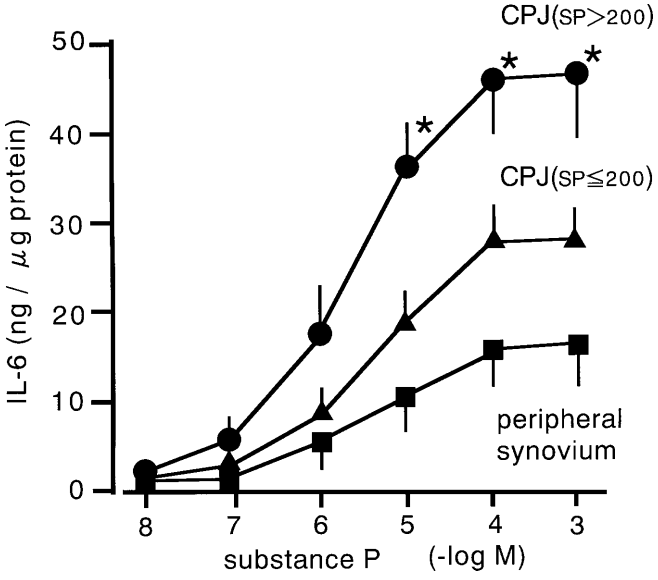

Fig. 2. Dose–response curves of substance P-induced IL-6 production by cultured synovial cells from the CPJ and peripheral synovium. The IL-6 production was assessed as described in the text. Each symbol represents the mean ± SE of 30 preparations. $*P < 0.01$ compared with the peripheral synovium

Discussion

RA is a chronic disease characterized by inflammation, altered immune responses, and synovial hyperplasia, resulting in the progressive destruction of affected joints.[8,9] We previously described the binding of synovial cells to adjacent cartilage and bone, which is a key mechanism involved in joint destruction, and which plays a central role in the pathophysiology of pannus formation in the CPJ of RA patients.[10]

The inflammatory neuropeptide substance P is involved not only in interactions of the nervous system and immune system,[11] but also in interactions of sensory C-fiber distribution in the synovium and in synovial pannus formation.[12,13] In addition, several studies have indicated that the production and release of inflammatory cytokines, such as IL-1β and IL-6 produced by synovial cells, are triggered by NK receptor activation.[14] IL-1β, in particular, increased the production of other inflammatory agents, such as collagenase and prostaglandin E_2, in synovial cells.[15] In this study, our analysis of the level of subsequent substance P-induced cytokine production in cultured synovial cells, and the concentration of substance P and cytokines in each part of the synovium, showed that existing substance P tended to be

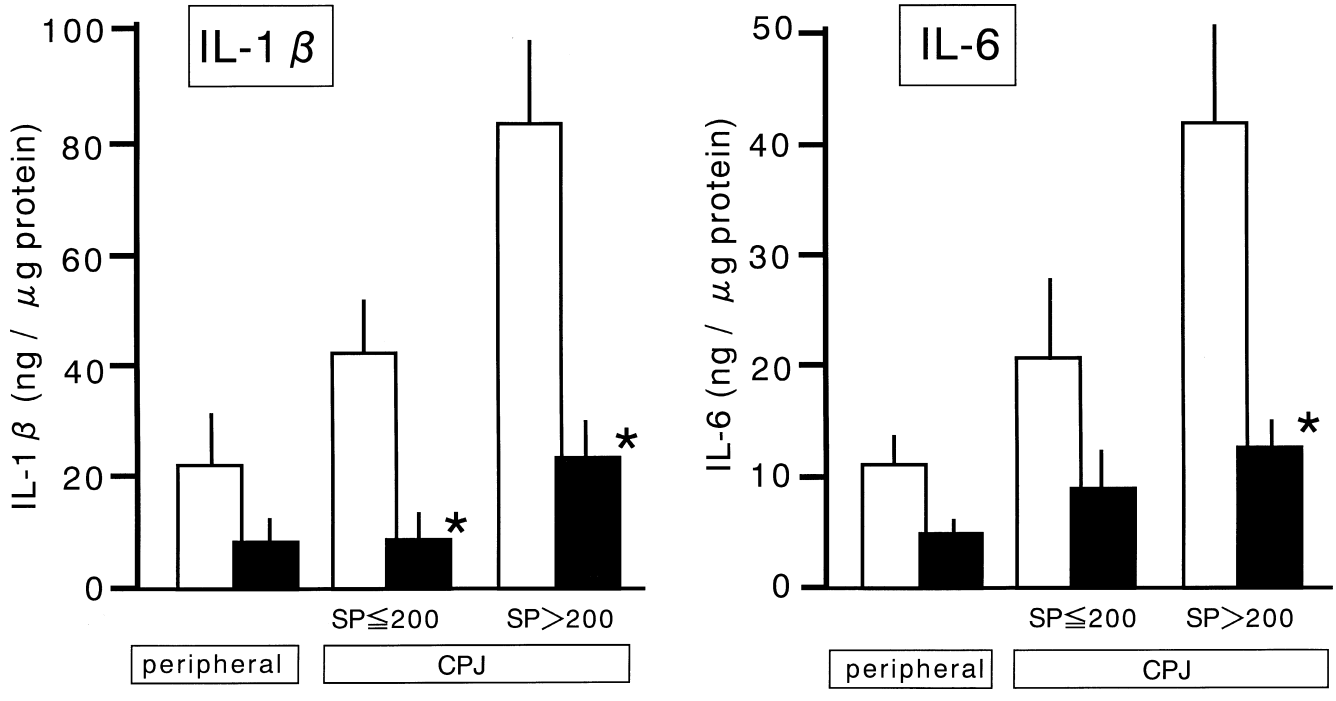

Fig. 3. Effects of the neurokinin-1 (NK-1) receptor antagonist GR82334 on substance P(30μM)-induced IL-1β and IL-6 production by cultured synovial cells from the CPJ and peripheral synovium. The IL-1β and IL-6 production (*open columns*: control) was significantly inhibited by GR82334 (1μM) treatment (*solid columns*) as described in the text. Each column represents the mean ± SE of 30 preparations. $*P < 0.01$ compared with the control synovium

more pronounced in individuals with higher levels of cytokine production. Since the cytokine production in response to substance P was clearly diminished in the peripheral synovium, we compared the effects of substance P between the CPJ and peripheral synovium according to the levels of cytokine production. Siginificant differences were found between the CPJ and peripheral synovium, and the trend towards a higher effect in the CPJ was increased. However, there was no difference in subsequent substance P ($0–10^{-8}$M) stimulation between the CPJ and peripheral tissue. If the existance of substance P is the potential cause of this difference, the difference would be observed in cultures with subsequent substance P stimulation ($0–10^{-8}$M). This difference was caused by the reactiveness of synovial cells which had previously been exposed to the existing concentration of substance P, and was not generated directly by the difference in the amount of substance P. We previously performed substance P synovial cell priming, analyzed the mechanism, and reported our findings.[16] Therefore, the results of this study were considered to be due to the priming effect of substance P, suggesting that the increased substance P-responsiveness of synovial cells from CPJ synovium could influence the magnitude of the effect of substance P in RA pannus formation. The culture cells obtained from four of seven patients who showed marked increases in substance P had significant levels of IL-1β and IL-6 induced by substance P stimulation, suggesting that other factors are involved in these substance P immunoregulatory systems. Since the marked increase observed in the CPJ was found at a substance P concentration of 10^{-6}M, these patients may have had increased sensitivity and/or responsiveness to the neuropeptide due to an alteration in the number and/or affinity of substance P receptors, or changes in intracellular signal transduction.

In summary, our findings indicated that cells containing substance P increased the cytokine production response in the CPJ synovium. However, some individual patients with RA did show a marked increase in the cytokine production-increasing effects of substance P, suggesting that substance P plays a role in the pathogenesis of RA via the upregulation of cytokine production. We consider that the existence of the priming effect of substance P was clarified by the analysis of the response of IL-1β production to the addition of substance P, as described above. Therefore, we should investigate the reason why an increase in substance P induces the priming effect that occurs in part of the CPJ. Of course, an increase in substance P might increase secondarily as a result of the inflammation reaction in each limited area of synovial tissue. However, it has already been proved that substance P is released with various stimuli, such as mental stress or pain from the nerve ending of sensory C-fiber. Other reports have indicated that there is a relationship between mental stress and joint inflamation in rheumatoid arthritis patients. We consider that the rise in the substnce P density is absorbed first by stimulation, such as mental stress. Further studies are required on the mechanism of this increase in the existing substance P density, and on the subsequent substance P-induced cytokine production response.

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