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Substance P Induces the Secretion of Gelatinase A from Human Synovial Fibroblasts

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Summary: We investigated the secretion of the matrix metalloproteinases, interstitial collagenase (matrix metalloproteinase-1), gelatinase A (matrix metalloproteinase-2) and stromelysin-1 (matrix metalloproteinase-3) in human synovial fibroblasts after stimulation with the neuropeptide substance P.

Human synovial fibroblasts were stimulated with substance P or interleukin-1β (IL-1β). In the cell culture media gelatinase A, interstitial collagenase and stromelysin-1 were identified and their activities towards different substrates were determined.

Substance P in synovial fibroblasts induced an increase in the overall matrix metalloproteinase activity towards the dinitrophenyl-labelled peptide by 85%, against an increase of 124% after stimulation with IL-1β. In case of substance P stimulation, the increase in activity reflects a significantly enhanced secretion of gelatinase A, whereas no significant increase of stromelysin-1 and collagenase secretion could be observed.

The matrix metalloproteinase pattern showing the highest gelatinase A secretion was obtained after stimulation with substance P. This pattern was very pronounced and differed very clearly from the pattern seen after IL-1β stimulation which caused a significant rise in collagenase and stromelysin-1 activity. We assume that distinct stimulation pathways are involved and that the neuropeptide (substance P), which is always present in the inflamed joint, plays its own and separate role in proliferative processes leading to the cartilage destruction.

Introduction

During the last years the possible role of neuropeptides, especially substance P in rheumatoid arthritis, has been discussed. Substance P is implicated in neurogenic inflammatory processes, induction of vasodilatation, oedema and pain (1, 2).

High levels of substance P – not produced by synoviocytes, but rather of neurogenic origin – are found in rheumatoid synovial fluid (3–7). After application of substance P in vitro, the release of interleukin-1 and interleukin-6 from monocytes as well as oxidative enzymes from macrophages are observed. *Lotz* et al. (8) also report on secretion of collagenolytic activity by monocytes after substance P treatment.

Inconsistent reports exist whether or not proliferation of synovial fibroblasts occurs after stimulation with substance P (5, 9, 10). It is also documented that the neuropeptide substance P induces the production of prostaglandin E_2, interleukin-1 and collagenase in synovial fibroblasts (5, 11). In these cells, but also in monocytes/macrophages and leukocytes, which are all present in the inflamed joint, not only collagenase, but also members of the three subclasses of matrix metalloproteinases – collagenase, gelatinase A and stromelysin-1 – are expressed. In non-inflammatory situations these enzymes[1]) are responsible for normal remodeling of the extracellular matrix. Their activities are strictly regulated by activation and inhibition processes. However, the key position of regulation remains to be the induction of matrix metalloproteinase expression (12), which is under the control of several cytokines, growth factors, tumour promoters and oncogenes (13). Only substances like the second messenger analogue of diacylglycerol,

[1]) Enzymes:
Fibroblast collagenase (Matrix metalloproteinase 1) EC 3.4.24.7
Gelatinase A (Matrix metalloproteinase 2) EC 3.4.24.24
Gelatinase B (Matrix metalloproteinase 9) EC 3.4.24.35
Stromelysin-1 (matrix metalloproteinase 3) EC 3.4.24.17
Trypsin EC 3.4.21.4

phorbol-12-myristate-13-acetate, have a more uniform stimulatory effect on the expression of all matrix metalloproteinases in various tissues (13). Interleukin-1 induces selectively the production of collagenase and stromelysin-1 in synovial fibroblasts (14–16). Tumour growth factor β on the other hand induces selectively the gelatinases A and B, while it has an inhibitory effect on collagenase and stromelysin-1 expression (13).

Considering the action of substance P, *Lotz* et al. (5) suggested a more general stimulatory effect on collagenase expression as part of an overall increase in protein synthesis.

Here we report on the pattern of matrix metalloproteinase expression (gelatinase A, interstitial collagenase and stromelysin-1) after stimulation of synovial fibroblasts with substance P. The substance P-induced matrix metalloproteinase pattern is compared to that obtained after stimulation with interleukin-1, the central mediator of inflammation.

Materials and Methods

Materials

Synovial tissue originated from 20 patients with rheumatoid arthritis of the Rheumaklinik, Immanuel-Krankenhaus, Berlin, undergoing surgical synovectomy or total joint replacement. None of these patients had obtained any steroidal antiinflammatory therapy, but they received other therapies of various kinds.

The following materials were purchased: human recombinant IL-1β from Biomol, Hamburg, Germany; N-(2,4)-dinitrophenyl-Pro-Gln-Gly-Ile-Ala-Gly-Gln-*D*-Arg (dinitrophenyl-labelled peptide) from Bissendorf Biochemicals, Hannover, Germany; the fluorogenic substrates (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu[3-(2,4-dinitrophenyl)-*L*-2,3-diaminopropionyl]-ala-Arg-NH_2 and (7-methoxycoumarin-4-yl)acetyl-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(2,4-dinitrophenyl)-NH_2 and substance P from Bachem, Heidelberg, Germany; *Dulbecco*'s modified *Eagle*'s medium, fetal calf serum, *Hank*'s balanced salt solution, penicillin/streptomycin, trypsin/EDTA and amphothericin B from Biochrom, Berlin, Germany. All other substances were obtained from Sigma Chemicals, Deisenhofen, Germany.

Gelatinase A and stromelysin-1 were isolated in our laboratory as previously described (17, 18). Gelatinase B was purified from buffy coat essentially as described (19). Collagen I was prepared from calf skin according to the method of *Miller* et al. (20).

Cell culture

Human synovial tissue obtained from patients with rheumatoid arthritis was enzymatically digested, and adherent cell cultures were established. The synovial cells were maintained in *Dulbecco*'s modified *Eagle*'s medium containing fetal calf serum (volume fraction 0.1) until confluency was achieved. According to *Firestein* et al. (21), in primary cultures of the synovial lining cells after 2 or 3 passages a relative homogenous population of fibroblasts like synoviocytes is left. So in the 3rd and 4th passages, the culture medium was changed to *Dulbecco*'s modified *Eagle*'s medium 2 g/l lactalbumin hydrolysate, and the cells were stimulated with substance P (10^{-8} mol/l) and interleukin-1β (0.3 μmol/l). After 24 h the media were collected. To overcome the variability of the cell cultures of the different donors as well as the variability within the group of culture flasks of one single donor, the activity increases of the matrix metalloproteinases of a single cell culture dish was always related to the values obtained before stimulation and not to the parallel control cultures.

Enzyme assays

General matrix metalloproteinase-activities were assayed with the synthetic substrate dinitrophenyl-Pro-Gln-Gly-Ile-Ala-Gly-Gln-*D*-Arg (dinitrophenyl-labelled peptide, 250 mg/l) as described by *Masui* et al. (22) and with the fluorogenic substrate (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu[3-(2,4-dinitrophenyl)-2,3-diaminopropionyl]-Ala-Arg-NH_2 (25 μmol/l) as introduced by *Knight* et al. (23).

The stromelysin-1 specific fluorogenic substrate (7-methoxycoumarin-4-yl)acetyl-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(2,4-dinitrophenyl)-NH_2 (12.5 μmol/l) was employed essentially as described by *Nagase* et al. (24).

In the three assays 50 μl of the medium samples, 5 μl 40 mmol/l *p*-aminophenylmercury acetate (APMA) in dimethyl sulphoxide and 45 μl 50 mmol/l Tris-HCl, pH 7.0, 200 mmol/l NaCl, 5 mmol/l $CaCl_2$, 5 μmol/l $ZnCl_2$, 0.5 g/l NaN_3 were incubated with 100 μl of the distinct substrate solution.

In the fluorescence assays the activities were continuously monitored in microtitre plates for 90 min at 25 °C. In the assay with the dinitrophenyl-labelled peptide the reaction was stopped after 5 h with 0.5 ml 0.5 mol/l HCl. The hydrolyzed dinitrophenyl-labelled peptide was extracted with acetic ethylester/butanol (1 + 0.15, by vol.), and the absorbance determined at 365 nm.

Collagenase activity was also measured with type I collagen as substrate. Matrix metalloproteinase solutions (20 μl) were activated with 2 mmol/l mersalylic acid and incubated with 20 μl 2 g/l collagen I for 20 h at 20 °C. Degradation of collagen I was analysed by 8% SDS-PAGE.

To demonstrate the presence of the progelatinase A-TIMP-2 complex,[2]) its inhibitory capacity against active gelatinase B was shown. The media were ultrafiltrated at a relative molecular mass cut-off of M_r 30 000 to eliminate TIMP-1. The gelatinase A-TIMP-2-containing fractions were incubated with active gelatinase B for 30 min at 37 °C and the activity of gelatinase B was measured using the dinitrophenyl-labelled peptide (22) as substrate (incubation: 30 min, 37 °C).

Gelatin zymography

Twenty (20) μl of culture media were prepared without heating or reduction for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 2 g/l gelatin. After electrophoresis the gels were washed twice in 25 g/l Triton X-100 in 50 mmol/l Tris-HCl pH 7.6, 200 mmol/l NaCl, 5 mmol/l $CaCl_2$, 5 μmol/l $ZnCl_2$, 0.5 g/l NaN_3, then they were washed shortly in the same buffer without Triton. The gels were incubated for 6 h at 37 °C in the same buffer containing 10 g/l Triton X-100. Afterwards they were fixed and stained in Coomassie Brilliant Blue.

Western blot

Immediately after electrophoresis, proteins were blotted to Immobilon membranes. Immunologic blot analysis was performed using affinity purified polyclonal rabbit anti-human-gelatinase A (1 : 500), polyclonal guinea pig anti-rabbit-collagenase (1 : 200) and guinea pig anti-rabbit-stromelysin-1 (1 : 1000). The second antibodies used were goat anti-rabbit-IgG-peroxidase conjugate and rabbit anti-guinea pig-IgG-peroxidase conjugate (both 1 : 2000). The blots were developed with aminoethyl carbazole.

Extraction of protein bands after SDS-PAGE

Sodium dodecylsulphate gel electrophoresis (10% SDS-PAGE) was performed as generally established. To relate single matrix metalloproteinase activities the respective protein bands were ex-

[2]) TIMP = Tissue inhibitor of metalloproteinases

cised from the gel after a very short staining with water soluble Coomassie Brilliant Blue. For protein extraction the sections were placed into separate tubes each containing 200 µl 50 mmol/l Tris-HCl, pH 7.0; 200 mmol/l NaCl, 5 mmol/l $CaCl_2$, 5 µmol/l $ZnCl_2$, 0.5 g/l NaN_3, 10 g/l Triton X-100 followed by an overnight incubation at room temperature. Matrix metalloproteinase-activities were determined as described before to obtain a relative pattern of activities.

Results

Generally

The total activity of metalloproteinases towards the dinitrophenyl-labelled peptide determined in the culture media of primary synovial fibroblasts as compared to the activities of the single culture dish before stimulation is increased by more than 124% 24 h after application of IL-1β. However, the exposure to the neuropeptide substance P (10^{-8} mol/l) induces an increase in matrix metalloproteinase activity of about 84% (fig. 1). Unstimulated cells show a spontaneous augmentation of matrix metalloproteinase activity of about 27% after changing the medium from fetal calf serum to lactalbumin hydrolysate. In gel electrophoresis (fig. 2) the media of cells treated with substance P show a strikingly different protein pattern compared to that obtained after interleukin-1 stimulation. Indeed, gelatinase A at M_r 67 000 can be seen as a clear protein band after substance P exposure, whereas after interleukin-1β treatment this band is slightly decreased. However, the secretion of collagenase and stromelysin-1 (four bands closely positioned at about M_r 50 000) is barely enhanced after substance P treatment, but appear very pronounced after interleukin-1 treatment.

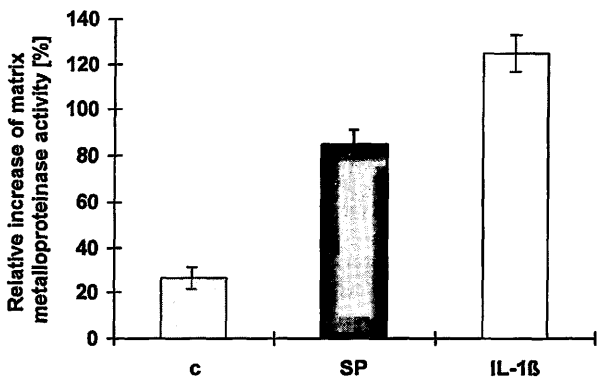

Fig. 1 Relative increase of overall matrix metalloproteinase activity secreted by synovial fibroblasts 24 h after stimulation with substance P (SP, 10^{-8} mol/l) and interleukin-1β (IL-1β, 0.3 µmol/l).
Samples were activated with 2 mmol/l p-aminophenylmercury acetate. The activity was measured with the dinitrophenyl-labelled peptide after overnight incubation at 37 °C. The activities determined in all media samples before withdrawal of fetal calf serum were defined to be 0;
c = parallel control without stimulation, but with change to lactalbumin hydrolysate containing medium.

Gelatinase A-TIMP-2 complex

The fact that gelatinase A is the predominant enzyme after substance P treatment, but faints after interleukin-1 exposure is demonstrated by gelatin zymography (fig. 3) and immunoblot analysis (fig. 4c). It is known that human synovial fibroblasts secrete progelatinase A mainly complexed with the tissue inhibitor[2]) of metalloproteinases TIMP-2. After activation the whole complex displays gelatinolytic activity, while progelatinase A-TIMP-2 is an inhibitor of other matrix metalloproteinases as well (17). TIMP-2 is visible in gel electrophoresis only after silver staining (not shown). After ultrafiltration the protein fraction greater than M_r 30 000 of substance P treated media inhibits gelatinase B three times more than the control media.

These results are consistent with the matrix metalloproteinase activities we measured in the extracts of the gel-eluted protein employing a fluorogenic matrix metallo-

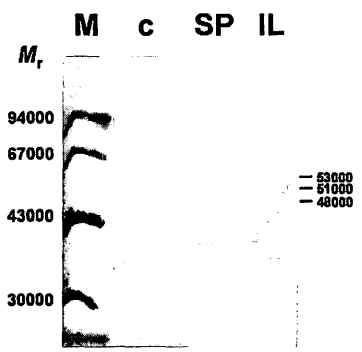

Fig. 2 10% SDS polyacrylamide gel electrophoresis of culture media of human synovial fibroblasts without stimulation (control, c) and after stimulation with substance P (SP) or interleukin-1β (IL). Twenty (20) µl of 5-fold concentrated media were applied to each lane. The gels were run under non-reducing conditions and stained with Coomassie Brilliant Blue.
Lane M: Molecular mass markers (M_r): phosphorylase b (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000).

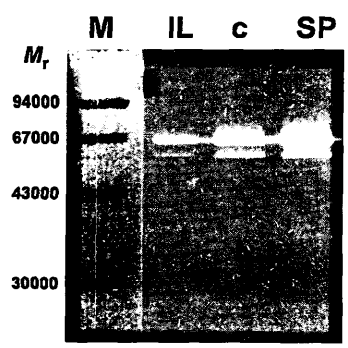

Fig. 3 Zymogram analysis of gelatinase A secreted into culture media by human synovial fibroblasts without stimulation (control, c) and after treatment with interleukin-1β (IL) and substrate P (SP). Medium samples were assayed 24 h after stimulation. The active M_r 67 000 form and the further activated M_r 62 000 form of gelatinase A are visible.
Lane M: Molecular mass markers (M_r): phosphorylase b (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000).

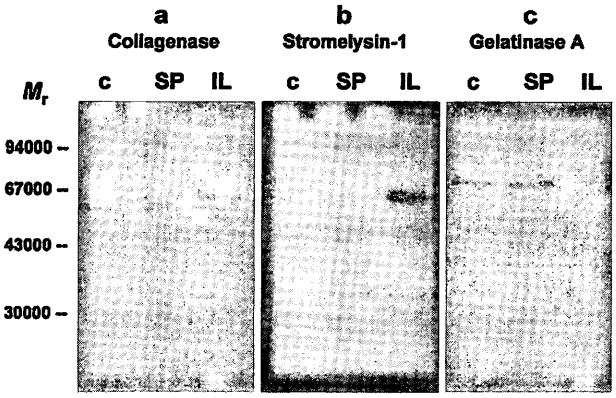

Fig. 4 Immunoblot of culture media of human synovial fibroblasts.
c: control media; SP: after stimulation with substance P; IL: after stimulation with interleukin-1β. Blot a was incubated with guinea pig anti-rabbit interstitial collagenase. Blot b was incubated with guinea pig anti-rabbit stromelysin-1. Blot c was incubated with rabbit anti-human gelatinase A.

proteinase substrate introduced by *Knight* et al. (23). After substance P treatment the protein eluted from the band at M_r 67 000 displays a gelatinolytic activity of 1.13 mU per band, in contrast to 0.74 mU per band in the control. The treatment with interleukin-1 resulted in a significant decrease of the gelatinase activity to 0.06 mU per band.

Interstitial collagenase

A typical electrophoretical pattern of media after stimulation with the neuropeptide substance P or the cytokine interleukin-1 is presented in figure 2. It is evident that after substance P exposure the intensities of the protein bands around M_r 50 000 do not differ significantly from the protein bands seen in the control media. However, interleukin-1β treatment induces a remarkable increase of interstitial collagenase, which is very pronounced in the M_r 51 000 form of this collagenase.

The collagenase activity of the extracts derived from the gel section was estimated by virtue of cleaving collagen type 1 (fig. 5). An identical observation can be made using either the unstimulated media (lane 2, 3) or the media after exposure to substance P (lane 4, 5). The protein extracts of the band at M_r 53 000 and M_r 48 000, which represent the latent and active forms of interstitial collagenase both hydrolyse moderately collagen type I. After interleukin-1 treatment the activity of the M_r 53 000 collagenase is not different from that observed in the media of substance P treated and control cells (lane 2–5). But an additional and rather broad band seen at M_r 51 000 as well as the M_r 48 000 collagenase, which represents only a rather weak band in gel electrophoresis (lane 7, 8), cleave collagen type I.

Western blot analysis (fig. 4a) of the media shows that anti-collagenase binds predominantly to the triple band of collagenase. In the interleukin-1β stimulated media especially the M_r 53 000 band is very pronounced (fig. 4a, lane 3). After exposure to substance P the three bands are only slightly more distinct (lane 2) than in the control media. The active M_r 48 000 form seems to be equally recognized by the antibody in all three groups.

Stromelysin-1

According to Western blot analysis stromelysin-1 seems to be increased only slightly after substance P treatment (fig. 4b, lane 2), but the high rate of secretion after IL-1 stimulation is by far not reached (lane 3).

Trying to identify the stromelysin-1 band which possibly overlaps with collagenase bands, we extracted four gel slices of M_r 53 000, M_r 51 000, M_r 50 000 and M_r 48 000 from sodium dodecylsulphate gels following water soluble Coomassie staining. Stromelysin-1 could only be detected in media exposed to interleukin-1 at about M_r 50 000 by its ability to hydrolyze a specific fluorogenic substrate introduced by *Nagase* et al. (24). The stromelysin-1 activity was about 0.05 ± 0.022 mU/band.

Discussion

In the present study, we analysed the secretion pattern of the interstitial collagenase (matrix metalloproteinase-1), stromelysin-1 (matrix metalloproteinase-3) and gelatinase A (matrix metalloproteinase-2) in culture media of synovial fibroblasts, untreated and treated with the neuropeptide substance P and the cytokine interleukin-1β.

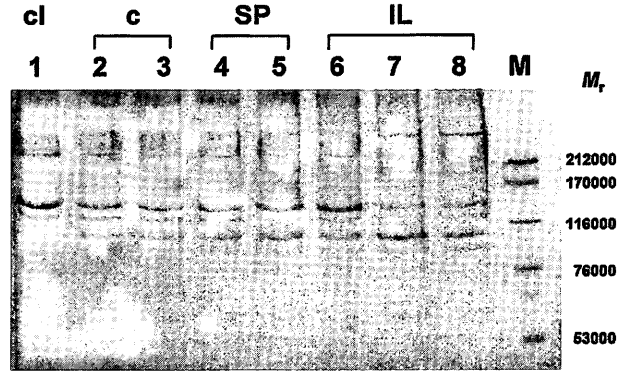

Fig. 5 Collagenolytic activity against collagen type I: After polyacryl amide gel electrophoresis of synovial fibroblast culture media the separated matrix metalloproteinases were excised from the gel, extracted and incubated with collagen type I (cI). Lane 1 shows the collagen type I control, Lane 2 and 3: collagen type I after incubation with M_r 53 000 and M_r 48 000 collagenase from the control media (c); Lane 4 and 5: collagen type I after incubation with M_r 53 000 and M_r 48 000 collagenase after substance P-treatment of cells (SP); lane 6–8: collagen type I after incubation with M_r 53 000, M_r 51 000 and M_r 48 000 collagenase as obtained after stimulation of cells with interleukin-1β (IL).
Lane M: Molecular mass markers (M_r): myosin (212 000), α_2-macroglobulin (170 000), β-galactosidase (116 000), transferrin (76 000), glutamic dehydrogenase (53 000).

Although the matrix metalloproteinase activities were measured in the culture media of cells of different donors the activity increases obtained (expressed in % in relation to the activities before stimulation) were homogenous and the observed differences significant. The donors were not under any steroidal antiinflammatory therapy, but indeed under various other medication and in different stages of the disease. Nevertheless, it can be assumed that at the time of stimulation (after 3 to 4 passages) the cells were no longer exposed to the original inflammatory cytokine milieu, and a relative homogenous population of synovial lining cells in a quiescent state were present (21).

In the gelatin zymography (fig. 3) the media of substance P treated cells display not only a remarkable increase of the M_r 67 000 form, but also of the activated M_r 62 000 form of gelatinase A. This observation is quite in contrast to the situation seen in the media of interleukin-1 treated cells, where a decrease in gelatinase A activity can be observed. It seems that substance P – if at all – has only a moderate effect on the induction of stromelysin-1 and collagenase secretion, whereas both matrix metallproteinases are predominantly present in the media of cells exposed to interleukin-1 (figs. 2 and 4). *Lotz* et al. (5) who did not determine the activities of gelatinase and stromelysin-1 separately, found a 5 to 8-fold increase in collagenolytic activity against collagen type III. We did not find such a remarkable increase after substance P treatment. The discrepancy may be due to the different assays applied. Moreover, in the assay of *Lotz* et al. (5) it is not clear, to what extent stromelysin-1 and gelatinase A are involved in the observed degradation of collagen type III.

As shown here the substance P induced increase of the overall matrix metalloproteinase activity (85%) towards the dinitrophenyl-labelled peptide is mainly due to enhanced gelatinase A expression.

The selective upregulation of gelatinase A has also been reported for transforming growth factor-β (13), but simultaneously collagenase and stromelysin-1 were downregulated. In cultured fibroblasts transforming growth factor-β also induces the expression of gelatinase B (13). We definitely did not observe any gelatinase B activity (fig. 3, zymogram) after substance P stimulation. However, collagenase and stromelysin-1 activity were not significantly enhanced.

While the regulation of collagenase and stromelysin-1 gene expression have been extensively investigated, the regulation of gelatinase A gene expression is by far not clear. But it is known that the oncogene families fos and jun are involved in positive as well as in negative regulation of matrix metalloproteinase-expression. Phorbol-12-myristate-13-acetate and the cytokines interleukin-1β and tumour necrosis factor-α as well as transforming growth factor-β induce c-fos gene expression, but finally display opposite effects in matrix metalloproteinase-expression (13). The neuropeptide substance P represents a different type of stimulus which strongly induces gelatinase A and does not significantly affect collagenase and stromelysin-1 secretion.

Lotz et al. (5) postulated a general increase in total protein synthesis, including collagenase synthesis. Our data indicate that the postulated augmented protein expression – at least within the matrix metalloproteinases – was not at all general, but rather significantly shifted mainly to gelatinase A (fig. 2).

Lotz et al. (5) also showed that substance P and interleukin-1 stimulation of fibroblasts occurs through distinct receptors. Substance P is known to act via receptors of the neurokinin-1-type in epithelial cells (25). *Oblas* et al. (26) report of an substance P binding protein from the heat shock (HSP) 70 family in horse salivary glands. Heat shock substances are among others also known to stimulate matrix metalloproteinase-expression in different cell types (for review see l. c. (13)). The receptor implicated in the stimulation of synovial fibroblasts has so far not yet been identified.

Tissue responses to the exposure to substances such as lipopolysaccharides or phorbol esters are often amplified by autocrine cytokine expression (15). A significant release of prostaglandin E_2 and interleukin-1 resulting from substance P treatment of synovial fibroblasts has been observed by several authors (5, 27). Cytokine interleukin-1 is known to induce not only matrix metalloproteinase-secretion, but also the expression and secretion of prostaglandin E_2 (28). Therefore, one might expect that a prolonged exposure of synovial cells to substance P may lead to an enhanced collagenase and stromelysin-1 secretion via a substance P-induced upregulation of interleukin-1 or prostaglandin E_2. Treatment of cells for 24 h was obviously not sufficient to observe this effect. On the other hand it was demonstrated (29) that the interleukin-1 effect on fibroblast collagenase-expression is not dependent on enhanced prostaglandin E_2 production, but that both mediators increase collagenase expression by separate regulatory mechanisms (30, 31).

The neuropeptide substance P seems to display multiple effects in the inflamed joint. Substance P induces mainly gelatinase A. Apart from its gelatinolytic activity this matrix metalloproteinase has an invasive potency to cells, which may enable them to destroy matrix components. In the state of chronic inflammation and pain this invasive potential may promote the proliferative processes of the synovial fibroblasts, the formation of pannus and its ability to invade the cartilage and to destroy bone. Furthermore, at the same time the neuropeptide

induces the secretion of cytokines from monocytes, macrophages and synoviocytes which are all present in the inflamed joint (5, 9, 29) and further enhance the vicious circle of inflammation and tissue destruction.

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