In Situ Hybridization of Stromelysin mRNA in the Synovial Biopsies from Rheumatoid Arthritis

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We examined the expression of stromelysin mRNA (SL mRNA) in synovial biopsy specimens from 12 cases of rheumatoid arthritis (RA) and 2 cases of osteoarthritis (OA) using in situ hybridization. The study demonstrated that positive cells with high levels of SL mRNA were mostly (85%) found in the synovial lining layer. The positive cells were abundant in the synovium of RA which presented well developed lymphoid follicles with massive inflammatory cells. On the other hand, the synovium of OA contained no positive cells for SL mRNA. In addition, low yet positive levels of SL mRNA were detected in the endothelial cells and vascular myocytes, and interstitial cells in the deeper layer of the synovium. Karyometric studies showed that cells positive for SL mRNA had significantly larger and more spherical nuclei than weakly positive or negative cells. The SL mRNA positive cells did not demonstrate any immunoreactivity to markers of bone marrow origin, such as Leu M1, Leukocyte Common Antigen (LCA) and lysozyme antigen. Electron microscopy of a case with many SL mRNA positive cells showed that most had well developed rough endoplasmic reticulum and numerous processes on the cell surface, and some had also well developed rough endoplasmic reticulum but without processes indicating that they may be AB and/or B synoviocytes. ——— rheumatoid arthritis; stromelysin; in situ hybridization; immunohistochemistry

The metalloproteinases, collagenase, gelatinase and stromelysin are involved
in degradation of extracellular matrix (Krane 1982). Collagenase cleaves molecules of collagen types I, II and III at a single locus (Okada et al. 1987) and those of type X collagen at two loci (Schmidt et al. 1986). Gelatinase can cleave denatured collagen (gelatin) and collagen types IV and V (Murphy et al. 1985). Stromelysin has the widest substrate specificity of all and degrades fibronectin, laminin, collagen type IV (Hoyhtya et al. 1988) and IX (Okada et al. 1989a), and cartilage proteoglycan (Galloway et al. 1983). These proteinases also act as an endogenous proactivator for procollagenase (Murphy et al. 1987). Prostromelysin, with a molecular weight of 51–55 kDa, is converted to an active form with a molecular weight of 45–48 kDa and another, with molecular weight of 24–28 kDa (Murphy et al. 1986). Stromelysin is now considered to be the same molecule as matrix metalloproteinase-3 (MMP-3), transin and metalloendopeptidase, proteoglycanase and collagenase activator (Murphy et al. 1988).

Several different human cell lines are capable of synthesizing stromelysin following induction with chemical agents (Aggeler et al. 1984). A number of factors are known to activate stromelysin including interleukin-1 (IL-1) (Quinones et al. 1989), epidermal growth factor (EGF) (Matrisian et al. 1986a) or tumor necrosis factor (TNF) (Dayer et al. 1985); conversely, inhibition of stromelysin occurs with tissue inhibitor of metalloproteinase (TIMP) (Gavrilovic et al. 1987).

Rheumatoid arthritis (RA) involves extensive destruction of matrix macromolecules in the joint (Harris 1985), and it is known that in this disease stromelysin as well as other proteinases (Harris et al. 1984) increase in the synovium. We report here on the expression of stromelysin (SL) mRNA in synovial biopsy specimens from 12 cases of RA and 2 cases of osteoarthritis (OA) using in situ hybridization. The results indicate that; (1) the synovial lining cells in RA contain high levels of SL mRNA; (2) the number of SL mRNA positive cells in the synovium correlates well with the histological features of the synovial tissue; (3) SL mRNA positive cells may belong to type AB and/or B synoviocytes; (4) SL does not accumulate in the synovial lining cells of patients with OA.

**Materials and Methods**

*Tissue preparation*

All synovial tissues used in this study were obtained from 12 RA patients at surgery for total knee replacement performed during 1987 to 1989 in the hospitals affiliating with Tohoku University School of Medicine, Sendai. All the patients were diagnosed according to the criteria of RA proposed by American College of Rheumatology in 1987 (Arnett et al. 1988). Tissues from two patients with osteoarthritis patients were included for comparison. The synovial specimens were fixed in a 4% paraformaldehyde (Polysciences, Inc., Warrington, PA, USA) in 0.01 M-phosphate buffer saline (PBS) at 4°C overnight and washed in PBS,
dehydrated in alcohol, and embedded in paraffin. Paraaffin sections cut at 3 μm thick were processed for in situ hybridization, immunohistochemistry and hematoxylin-eosin staining.

**In situ hybridization**

Hybridization procedures used in this study were essentially the same as described previously (Hayashi et al. 1986). Briefly, deparaffinized sections mounted on coated microscope slides were treated with pronase (0.25 mg/ml in 50 mM-Tris-HCl, pH 7.6 and 5 mM-EDTA) (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA) for 10 min and acetylated with a freshly diluted acetic anhydride (0.25% in 0.1 M-triethanolamine buffer, pH 8.0) for 10 min. The treated sections were processed for in situ hybridization at 45°C for 18 hr in a mixture containing the tritiated cDNA probe (1 μg/ml), yeast tRNA (500 μg/ml) (Sigma Chemical Co., St. Louis, Mo, USA), salmon sperm DNA (80 μg/ml) (Sigama Chemical Co.), 50% formamide (Bethesda Research Laboratories, Bethesda, MD, USA), 10 mM-Tris-HCl, pH 7.0, 0.15 M-NaCl, 1 mM EDTA, pH 7.0, Denhardt's mixture and 10% dextran sulfate (Pharmacia P-L Biochemicals, Piscataway, NJ, USA).

SL cDNA employed was a 0.3 k-base-pair EcoR1-Pvull fragment from H25A (Saus et al. 1988). Probes were labeled with 3H-deoxy thymidine triphosphate (3H-dTTP) (New Eng. Nucl., Boston, MA, USA) by nick translation to a specific activity of 2.5 × 10⁷ cpm/μg.

After hybridization and removal of the cover glass by immersing the slides in 2 × standard saline-citrate buffer (SSC) (1 × SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate pH 7.0) for 1 hr at room temperature, sections were washed in 2 × SSC three times and in 0.1 × SSC for 10 min each at 45°C. Afterwards, the slides were dehydrated in ethanol, dried in air, immersed in Kodak NTB-2 nuclear track emulsion and exposed for 14 days at 4°C. The exposed slides were developed in Kodak D-19 developer for 3 min at 18°C. The sections were stained with hematoxylin.

**Specificity of in situ hybridization to stromelysin mRNA**

The nucleotide sequence of SL cDNA shows 54% similarity to collagenase cDNA. Northern blotting under stringent conditions can clearly differentiate SL mRNA from collagenase mRNA (Saus et al. 1988).

The in situ hybridization protocol that we have employed allows differentiation of mRNAs, when the sequence similarity between the two molecule shows approximately 50% or less (Saus et al. 1988). In addition, sections digested with RNase (2 mg/ml, room temp.) (Sigama Chemical Co.) before in situ hybridization with the cDNA showed no labeling, implying that the hybridization with the probe was dependent on the presence of RNA in the tissue sections.
**Immunohistochemistry and histochemistry**

After deparaffinization by heat and in xylene and a rinse in 100% ethanol, sections were treated with 1% hydrogen peroxide in 50% methanol for 30 min to minimize endogenous peroxide activity and washed in PBS. The slides were then placed in a moist chamber and sections were covered with 5% normal rabbit serum in PBS for 30 min. The excess rabbit serum was removed by blotting and sections were covered with the primary antibody solution and incubated overnight at 4°C and rinsed in PBS. The primary antibody was a polyclonal antibody produced in the sheep, directed against human stromelysin (Okada et al. 1989b). The antibody to SL was used at a 1:1,600 dilution.

Sections were covered with the second antibody, biotin-labeled anti-sheep IgG (Vector Laboratories Inc., Burlingame, CA, USA) at a 1:50 dilution for 30 min at room temperature, rinsed in PBS and covered with avidin-biotin-antiperoxidase (ABC) (ABC kit; Vector Laboratories Inc.) at a 1:50 dilution for 30 min at room temperature and rinsed in PBS. Antigenic sites on sections were demonstrated by reacting the sections with a mixture of 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Grade II, Sigma Chemical Co.) in 0.05 M-Tris-HCl buffer, pH 7.6 and 0.01% hydrogen peroxide for 7 min. The sections were then counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene and mounted in Permount (Fisher Scientific Co., Fair Lawn. NJ, USA). Antibodies used were Leu M1 (Becton Dickinson, Mountain View, CA, USA) for myelo-monocyte; LCA (DAKO Japan Co. Ltd., Kyoto) for leukocyte common antigen and anti-lysozyme antibody (DAKO Japan Co. Ltd.). Negative controls for immunostaining were performed by replacing the primary antibody with the normal sheep serum.

Sections were also stained with 1% alcian blue dissolved in 3% glacial acetic acid (pH 2.5) or in 0.1 N hydrochloric acid (pH 1.0).

**Electron microscopy**

The synovial lining from Case 1 (see Table 1) was examined by transmission electron microscopy. The tissues were fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in graded alcohols, propylene oxide and embedded in Epon 812. One-μm thick sections were stained with toluidine blue and used to select areas for thin sectioning. Thin sections were stained with lead and uranyl acetate, mounted on copper grids, and examined in a JEOL 1200-EX electron microscope (Japan Electrooptics Laboratory Ltd., Tokyo).

**Karyometry**

Slides processed for in situ hybridization showed that with more than 10 silver grains per cell for SL mRNA were located in the superficial layer and that no such cells were present in the deepest layer. To quantify this observation, we
Table 1. Variability in the number of cells with high levels of stromelysin mRNA in relation to pathological features

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Duration (years)</th>
<th>Stage</th>
<th>Class</th>
<th>Histology</th>
<th>Number of cells with many grains</th>
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<td>55</td>
<td>F</td>
<td>9</td>
<td>IV</td>
<td>III</td>
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<td>Diffuse</td>
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<td>69</td>
<td>F</td>
<td>26</td>
<td>IV</td>
<td>IV</td>
<td>3+</td>
<td>Diffuse</td>
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<td>3</td>
<td>52</td>
<td>F</td>
<td>2</td>
<td>IV</td>
<td>IV</td>
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<td>Diffuse</td>
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<td>4</td>
<td>31</td>
<td>F</td>
<td>7</td>
<td>IV</td>
<td>III</td>
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<td>Diffuse</td>
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<tr>
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<tr>
<td>6</td>
<td>48</td>
<td>F</td>
<td>16</td>
<td>IV</td>
<td>III</td>
<td>1+</td>
<td>Fibrous</td>
</tr>
<tr>
<td>7</td>
<td>58</td>
<td>F</td>
<td>5</td>
<td>IV</td>
<td>III</td>
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<tr>
<td>8</td>
<td>64</td>
<td>F</td>
<td>8</td>
<td>IV</td>
<td>III</td>
<td>2+</td>
<td>Diff. + Fib.</td>
</tr>
<tr>
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<td>70</td>
<td>F</td>
<td>11</td>
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<td>IV</td>
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<td>Fibrous</td>
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<td>10</td>
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<td>18</td>
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<td>0</td>
<td>Fibrous</td>
</tr>
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</table>

Class and stage: “Steinbrocker’s classification” (Steinbrocker et al. 1949).
Lymph follicles: Degree of development of lymph follicles (3+, fully developed, 2+, moderately developed, 1+, slightly developed, 0, not detectable).
Interfollicles: Interfollicular features (Diffuse — diffuse inflammatory infiltration, Fibrous — fibrosis, Diff. + Fib. — both diffuse inflammatory infiltration with fibrosis.
Number of cells with many grains: Number of cells with more than ten autoradiographic silver grains for stromelysin mRNA per cell counted in 100 microscopic fields using 100× objective lens and 10× ocular lens.

divided the synovial tissues into four layers defined by the comparative depth from the surface to the deepest stromal area (A, >0 to 25 μm; B, >25 to 50 μm; C, >50 to 75 μm; D, >75 μm). We counted only the cells containing more than 10 silver grains per cell. The counting was performed in four areas randomly chosen from each layer of depth.

We also measured the long (a) and short (b) diameters of nuclei using an objective micrometer (Tezuka and Sawai 1983) and calculated the nuclear shape (ellipticity of nucleus) according to the following formula: 1) mean nuclear diameter, a+b/2; 2) nuclear area, π×a×b; 3) ellipticity; 1-b²/a². We compared between two groups of cells: one containing more than 10 grains per cell (SL mRNA positive cell) and the other, containing less than 4 grains per cell (SL mRNA weakly positive to negative cell). In each group, we measured 100 cells and analyzed the data by using the Student’s t-test.

Results

The location of cells with abundant SL mRNA in the synovial tissue of RA patients was predominantly in the lining cells.
Fig. 1 shows the distribution of cells which contained more than ten silver grains for SL mRNA at four progressive depths A, B, C, and D from the surface. Most (85%) of the positive cells were found in the most superficial layer (A) (Fig. 2). Moderate portion (12%) of positive cells were found in the next (B) layer.

![Distribution of positive cells](image)

**Fig. 1.** Number of SL mRNA positive cells in the synovium in four (A, B, C, D) layers of different depth in the synovium. Positive cells with many grains were mostly located in the most superficial layer (A).

![Synovial biopsy](image)

**Fig. 2.** Synovial biopsy of RA. In situ hybridization on paraffin sections using \(^3\)H-labeled cDNA encoding human SL mRNA and visualized by autoradiography. Positive cells containing many grains for SL mRNA are found in the synovial lining, with a considerable variation in the number of grains per cell (Case 1, Bar 25 \(\mu\)m).
The remaining (3%) positive cells were found in the next deeper layer (C). No positive cells were found in the deepest layer (D). Immunostaining of a comparable field, from slides of the same patient, with antibody to human stromelysin demonstrated a distribution of positive cells almost identical localization to that of mRNA demonstrated by in situ hybridization (Fig. 3).

Cells positive for SL mRNA were frequently found in the synovial tissue having massive inflammatory infiltrates and well developed lymphoid follicles, whereas they were rare or absent in the fibrotic or edematous synovial tissues with a few lymphoid follicles. On the other hand, there were no correlations between the number of SL mRNA positive cells and the patients' age, sex, duration of the illness, or the stage or class according to the classification proposed by Steinbrocker et al. (1949) (Table 1). In both the cases with OA, there was no cell that had more than ten grains in the synovium.

Cells positive for SL mRNA generally possessed large, spherical nuclei compared to the weakly positive to negative cells. Karyometry revealed that in the positive cells, the nuclear diameter ($D = 8.2 \pm 1.3 \mu m$) and nuclear area ($A = 213.1 \pm 64.3 \mu m^2$) were significantly larger ($p < 0.001$) and the nuclear ellipticity ($e^2 = 0.32 \pm 0.21$) was smaller than weakly positive or negative cells ($D = 7.3 \pm 1.3 \mu m$; $A = 150.1 \pm 54.8 \mu m^2$; $e^2 = 0.70 \pm 0.19$) (Figs. 4, 5 and 6). The synovial lining cells of OA patients showed their nuclear morphology ($D = 7.3 \pm 1.3 \mu m$; $A = 157.2 \pm 51.5 \mu m^2$; $e^2 = 0.67 \pm 0.15$) similar to those found in weakly positive or negative cells of RA patients. The synovial lining cells of RA patients did not exhibit any immunoreactivity to Leu M1, Leukocyte Common Antigen (LCA), or lysozyme antigen. Electron microscopy of a case which contained many positive cells for SL mRNA, showed that most cells in the synovial lining layer possessed

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Fig. 3. Immunostaining of the synovium with an antibody to stromelysin. Immunoreactive cells are found only in the lining layer (Case 1, Bar 25 $\mu m$).
well developed rough endoplasmic reticulum and a large number of cellular processes (Fig. 7), but that some cells did not have cytoplasmic processes.

The overall distribution of SL mRNA in synovial tissue, including synovial cells, endothelial cells, vascular myocytes, interstitial fibroblasts, lymphocytes, neutrophils and red blood cells, is illustrated in Fig. 8. We counted 100 cells for synovial cells, endothelial cells, and fibroblastic cells; 30 cells for myocytes and
Fig. 6. Nuclear ellipticity of positive cells with many grains (●●, ≥10 per cell) and weakly positive to negative cells with few grains (○○, <5 per cell). Smaller numeral indicates shape of a nucleus is more spherical (round).

Fig. 7. Ultrastructure of synovium in a case having a number of positive cells for SL mRNA (Case 1). A majority of cells in the lining show well developed rough endoplasmic reticulum and numerous cellular processes on the surface (Bar 5 μm).

lymphocytes; 10 cells for neutrophil leukocytes and red blood cells in 12 cases of RA and 2 cases of OA. The synovial cells contained many grains but with a wide range in number (8.9±8.1). Endothelial cells (2.0±0.9), interstitial fibroblast (1.5±0.7), and vascular myocytes (2.0±0.9) had only a small number of grains, and red blood cells (0.4±0.2) and neutrophils (0.5±0.2) background level. Labeling over vascular myocytes are illustrated in Fig. 9.
Fig. 8. The number of silver grains over various cell types in the synovium from 12 cases of RA and 2 cases of OA (see Text). Synoviocytes contain many silver grains for SL mRNA with a wide range in number. Endothelial cells and only myocytes contain small number of grains.

Fig. 9. Vascular myocytes expressing low levels of SL mRNA (Case 2, Bar 25 μm).
Synovial tissues from 2 cases of OA patients did not have any significant number of silver grains for SL mRNA: synovial cells (0.8±0.3), endothelial cells (0.8±0.6), interstitial fibroblasts (0.8±0.3), vascular myocytes (1.2±0.2), red blood cells (0.8±0.6).

Unexpectedly, when we examined a case having granulation tissues around deteriorating bones (pannus) including chondrocytes, osteoclasts and osteoblasts, we did not find any cells containing significant amounts of SL mRNA or protein.

**Discussion**

Stromelysin is a metalloproteinase and it has been known to have two major functions. One function of this enzyme is to cleave the several extracellular matrix molecules, i.e., laminin, fibronectin, type IV and IX collagen; the other function is that of an endopeptidase proactivator for procollagenase (Whitham et al. 1986; Frisch et al. 1987). In relation to the function of extracellular matrix degradation, stromelysin has a role in tumor invasion (Liotta et al. 1983). The mRNA coding for secreted protease transin is expressed more abundantly in malignant than benign tumors (Matrisian et al. 1986b). As an endopeptidase, collagenase activity is enhanced up to 12-fold by either natural or recombinant stromelysin in the presence of trypsin of 4-aminophenylmercuric acetate. A cascade mechanism is proposed in which collagenase is activated by stromelysin (Murphy et al. 1987). The expression of stromelysin is induced by IL-1, and is inhibited by dexamethasone, both at transcriptional levels (Quinones et al. 1989).

The results of this study indicate that most of SL mRNA in the synovial tissues of RA patients is accumulated in the synovial lining layer which was made up largely of synoviocytes. Since the superficial lining layer is facing the synovial cavity, the cells in this location are in close contact with the inducing substances, such as IL-1 and TNF present in the synovial fluid (Hopkins et al. 1988). At the same time, because synovial fluid contains not only the above activators but also some inhibitors such as tissue inhibitor of metalloproteinase (TIMP) (Cawston et al. 1984), this superficial position assures that synoviocytes exposing to the synovial fluid factors are controlled by them. Recent evidence indicates that the synovial cells themselves secrete IL-1 (Dalton et al. 1989), raising the likelihood that the synthesis of stromelysin in synovial lining cells can be activated by the cell's own product, IL-1, through an autocrine or paracrine mechanism (Brinckerhoff et al. 1989).

There was a wide difference in the number of positive cells among different synovial specimens and this difference was related to the histological features of the synovium. Synovial tissues presenting well developed lymphoid follicles and massive inflammatory infiltrates in interfollicular area contained many positive cells, while tissues presenting fibrotic or edematous features without mature lymphoid follicles contained few such cells. Histological features of synovial tissue correlated with the severity of RA (Rooney et al. 1988). In severely
inflamed synovium, there are many kinds of cells including synovial cells, lymphocytes, macrophages, neutrophils and fibroblasts. These cells produce cytokines, such as IL-1, TNF and platelet growth factor (PDGF) and γ-interferon, which enhance the production of metalloproteinase (Dunchan and Berman 1989; Kumkumian et al. 1989). In patients with RA, the severity of synovial inflammation as judged by immunohistochemical changes, arthroscopy and roentgen examination correlate well with the production of IL-1 by synovium (Miyasaki et al. 1988). TNFα is known to increase the amount of stimulated collagenase mRNA 5-fold (Scharffetter et al. 1989). IL-1 increases the expression of collagenase and stromelysin in cultured cells (Fig. 10). It is highly conceivable that the cytokines secreted from various inflammatory cells present in synovial tissue increase the synthesis of stromelysin in vivo.

Our study have shown that cells with a large number of silver grains for SL mRNA possessed a large, spherical nucleus compared with cells with few grains. Previous studies showed similar cell changes when procollagenase is induced by tumor-promoting phorbol diester (TPA) or cytochalasin B in rabbit fibroblasts (Werb et al. 1987). Disruption of actin cytokeratin by cytochalasin B produces similar spherical cell shape changes, which is associated with induction of metalloproteinase (Unemori and Werb 1962).

There are two types (A and B) or three types A, B and AB of synoviocytes in the lining layer (Linck and Porte 1981). Type A synoviocytes, or macrophage-like cells, have many cytoplasmic processes, abundant lysosomes, and demonstrates phagocytic activity (Mapp and Revell 1988). Type B synoviocytes or fibroblastic cells, have a number of rough endoplasmic reticulum (rER) and
secrete proteoglycans and glycosaminoglycans (Graabaek 1984). Type AB synoviocytes, or intermediate cells, show morphological features intermediate between A and B synoviocytes. The origin of these cells is not entirely clear. Type A cells are thought to be derived from monocytes in the bone marrow and type B cells from synovial mesenchymal cells (Howat et al. 1987). Furthermore, the two cell types can be transformed in vitro into one another under some conditions (Fell 1978). The SL mRNA positive cells showed no immunoreactivity to bone marrow cell markers nor to lysozyme. Electron microscopy of a case which contained many SL mRNA positive cells showed that most cells in the synovial lining layer contained both well developed rER and numerous cytoplasmic processes on the cell surface; these cells had few lysosomes or phagocytic features. However, some cells were devoid of cytoplasmic processes. The findings indicate that the majority of SL mRNA positive cells resemble AB synoviocytes with some resembling B synoviocytes. Further study is in progress to correlate in situ hybridization and ultrastructure on these cells. Recently Okada et al. (1989b) reported that cells responsible for synthesis of stromelysin have the phenotype of synovioblasts (B cells) by immunoelectron microscopy, but not of phagocytic synovial macrophage-like cells (A cells).

In vitro, several cell types such as endothelial cells, chondrocytes and fibroblasts are known to express stromelysin and collagenase in the presence of phorbol ester, IL-1 and TNF (Murphy et al. 1986). Our results showed that endothelial cells, vascular myocytes and fibroblasts had only low levels of SL mRNA. Immunostaining by Case et al. (1989) also demonstrated that stromelysin was expressed not only in synoviocyte but also in chondrocytes and endothelial cells of rheumatoid arthritis and in myocytes of rabbit. Wooley et al. (1977) reported the presence of collagenase in pannus-cartilage junction by indirect fluorescent study. We did not however find any cells with abundant SL mRNA in the pannus which developed in the lesions of severe bone destruction (Case 6). This interesting observation is being pursued in additional studies.

Acknowledgments

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