Synovectomy reduces stromal-cell-derived factor-1 (SDF-1) which is involved in the destruction of cartilage in osteoarthritis and rheumatoid arthritis

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We have compared the concentrations of stromal-cell-derived factor-1 (SDF-1), matrix metalloproteinase-1 (MMP-1), MMP-9 and MMP-13 in serum before and after synovectomy or total knee replacement (TKR). We confirmed the presence of SDF-1 and its receptor CXCR4 in the synovium and articular cartilage by immunohistochemistry. We established chondrocytes by using mutant CXCR4 to block the release of MMPs.

The level of SDF-1 was decreased 5.1- and 6.7-fold in the serum of patients with OA and RA respectively, after synovectomy compared with that before surgery. MMP-9 and MMP-13 were decreased in patients with OA and RA after synovectomy. We detected SDF-1 in the synovium and the bone marrow but not in cartilage. CXCR4 was detected in articular cartilage. SDF-1 increased the release of MMP-9 and MMP-13 from chondrocytes in a dose-dependent manner. The mutant CXCR4 blocked the release of MMP-9 and MMP-13 from chondrocytes by retrovirus vector.

Synovectomy is effective in patients with OA or RA because SDF-1, which can regulate the release of MMP-9 and MMP-13 from articular chondrocytes for breakdown of cartilage, is removed by the operation.

Synovectomy is a useful treatment for synovitis of the knee in the early stages of osteoarthritis (OA) or rheumatoid arthritis (RA) in order to relieve pain, but the mechanism by which it alleviates this remains to be clarified. In patients with OA and RA, arthroscopy often reveals synovial proliferation.¹ Synovectomy can provide safe and reliable results over the long term,² but we do not know the mechanism by which the synovial proliferation leads to destruction of cartilage. In joint fluid, there are many types of cytokines including chemokines, such as interleukin 1 (IL-1) and tumour necrosis factor (TNF) which induce destruction of cartilage and metalloproteinases (MMPs) in OA and RA.³ Recently, many types of chemokines such as MIP-1 and RANTES have been found in joint fluid produced by synovial tissue.⁴ We have already reported that one of the chemokine families, stromal-cell-derived factor-1 (SDF-1), in synovial fluid, may regulate the release of MMP-3 from cartilage in OA and RA.⁵ SDF-1 is mainly produced by macrophages and lymphoid cells to attract other lymphoid cells to the place of inflammation.⁴ In trying to compare the concentrations of SDF-1 after surgery, it is difficult to collect synovial fluid, especially after total knee replacement (TKR). We therefore decided to

measure the concentration of SDF-1 and MMPs in serum, which is easier to collect. Our hypothesis was that SDF-1 can be used as a marker of the efficacy of synovectomy. We measured the levels of SDF-1 before and after synovectomy in patients with OA and RA and studied the relationship between synovectomy and the release of MMPs.

Patients and Methods

SDF-1, MMP-1, MMP-9 and MMP-13 in serum before and after arthroscopic synovectomy or TKR. Serum was collected from patients diagnosed with osteoarthritis (OA) (n = 32; mean age 65 years; range 55 to 78) or rheumatoid arthritis (RA) (n = 28; mean age 68 years; range 48 to 79) before and after arthroscopic synovectomy. Serum was also collected from patients with OA (n = 31; mean age 71 years; range 65 to 79) or with RA (n = 30; mean age 73 years; range 67 to 82) before and after total knee replacement (TKR). All samples were collected from Gunma University Hospital and its affiliated hospitals in Japan. Patients with OA were diagnosed by radiography and arthroscopy and those with RA according to the criteria of the American College of Rheumatology.⁶ We measured the levels of SDF-1, MMP-1, MMP-9 and MMP-13 before and after arthroscopic syn-

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ovectomy or TKR (PFC Sigma Knee System, Depuy Leeds, UK). Patients undergoing synovectomy had relatively slight OA and RA of Larsen grade II or grade III,¹ with more joint space demonstrated on radiography compared with those who had had TKR. Hence, patients undergoing TKR had less proliferation of synovium than those undergoing synovectomy. The serum was centrifuged at 2 500 g for ten minutes to remove cells and debris and then stored at -80°C. The concentration of SDF-1 was quantified by a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instruction (Human SDF-1 α Immunoassay AN' ALYZATM, Minneapolis, Minnesota). The detection limit of the assay was 0.5 ng/ml for SDF-1. The activities of MMP-1, MMP-9 and MMP-13 were measured by ELISA according to the manufacturer's instruction (Biotrak; Amersham Bioscience, Piscataway, New Jersey).

Detection of SDF-1 in synovial tissue by immunofluorescent histology. For immunofluorescence staining, synovial tissue and articular cartilage were fixed at room temperature with 4% paraformaldehyde then embedded with paraffin. Each 5 μ m slice was mounted on a glass slide and washed with 100% xylene, 95% ethanol, 90% ethanol, 70% ethanol and phosphate-buffered saline (PBS) to remove paraffin. The slides were then washed with PBS and incubated with primary antibodies. An anti-human SDF-1 polyclonal antibody (R & D Systems Inc, Minneapolis, Minnesota) and a secondary antibody with conjugates (Jackson ImmunoResearch, West Grove, Pennsylvania) were used. After washing with PBS, affinity-purified donkey anti-mouse antibody (Jackson ImmunoResearch), was applied with or without Hoechst nuclear dye (0.5 mg/ml). The slides were washed and mounted in 95% glycerol in PBS. Single or multiple exposure photography was performed.

Detection of SDF-1 and its receptor, CXCR4, in cartilage using immunohistochemistry. In order to examine whether SDF-1 and its receptor CXCR4 are expressed in articular cartilage we performed immunohistochemistry, after removing paraffin from the sections, with anti-human SDF-1 mAb and anti-CXCR4 mAb (R & D Systems Inc.). We used a biotinavidin-horseradish peroxidase method (Histofine, Sab-Po(Multi) kit; Nichirei, Tokyo, Japan).

Chondrocyte and synovial cell culture and overexpression of mutant CXCR4 in chondrocytes. Pieces of articular cartilage were surgically excised from randomly chosen patients from each category during TKR, with informed consent. These were finely minced and chondrocytes were isolated by sequential enzymatic digestion at 37°C with 0.1% hyaluronidase for 30 minutes and 0.2% collagenase for one hour (Sigma, St Louis, Missouri). Isolated chondrocytes were filtered through 100 μ m nylon meshes, washed in Dulbecco's modified Eagles' medium, seeded at high density (5×10^4 /well) in 24-well plates and incubated at 37° in a humidified, 5% CO₂ atmosphere in an incubation medium of 10% fetal bovine serum in DMEM (Gibco BRL, Grand Island, New York), supplemented with 25 mM HEPES (Sigma), 100 IU/ml of penicillin (Biological Industries, Kib-

butz Beit Haemek, Israel), 100 mg/ml of streptomycin (Biological Industries), 50 mg/ml of gentamicin (Flow, Biaggio, Switzerland), and 2.5 mg/ml of amphotericin B (Biological Industries). These freshly isolated cells maintain their phenotype in primary culture as evidenced by a positive reaction to type-II collagen antibody and a negative reaction to type-I collagen antibody.

Cultured chondrocytes of 5×10^4 were seeded in 24-well plates with SDF-1 in concentrations of 0, 1, 10, 100 and 1000 ng/ml for two days. We then collected the supernatant to measure MMP-1, MMP-9 and MMP-13 by ELISA as described above. We also cultured synovial fibroblasts as follows. First, we cut synovial tissue from patients with OA and treated it with collagenase. The cells were cultured in DMEM. MMP-1, MMP-9 and MMP-13, and analysed after treatment with 0, 1, 10, 100 and 1000 ng/ml of SDF-1 for two days. More than 90% of the culture was composed of synovial fibroblasts.

We constructed the mutant CXCR4 by converting Asn-119 to Ser in transmembrane helix 3 in CXCR4, which is the important site for SDF-1/CXCR4 signalling in cells.⁷ Chondrocytes demonstrating overexpression of CXCR4 and mutant CXCR4 cells which expressed excessive CXCR4 and mutant CXCR4 were established as transfected with the plasmid encoding both the hygromycin-resistant gene and the receptor for amphotropic murine leukemia virus (MuLV) to isolate a sub-line positive for amphotropic MuLV receptors. Amphocotropic MuLV packaging cells were transfected by pMX-puro/CXCR4 and pMX-puro/mutant CXCR4 plasmids to make pseudotype viruses which had the amphotropic MuLV envelope.^{8,9} The cells resistant to hygromycin were then infected with the amphotropic MuLV pseudotype virus containing the PMX-puro/CXCR4 and the pMX-puro/mutant CXCR4 vectors, which codes for both CXCR4 or mutant CXCR4 and puromycin-resistant genes. The surviving cells were designated chondrocytes/mutant CXCR4. Chondrocytes/CXCR4 and chondrocytes/mutant CXCR4 were maintained in DMEM containing puromycin. MMP-1, MMP-9 and MMP-13 were measured by ELISA on day seven after transfection. The expression of the mutant CXCR4 gene in chondrocytes was checked by RT-PCR using primers specific to the full-length CXCR4 for DNA sequence. We also confirmed the protein level of mutant and full-length overexpression of CXCR4 by flow cytometry.

Statistical analysis. We used the Wilcoxon test to compare concentrations of SDF-1, MMP-1, MMP-9, and MMP-13 before and after surgical treatment and the Mann-Whitney U test to compare levels of MMP-1, MMP-9 and MMP-13 between control and mCXCR4 transfected cells. P values <0.05 were considered to be significant.

Results

SDF-1, MMP-1, MMP-9 and MMP-13 levels in serum before and after synovectomy or TKA. The mean concentrations of SDF-1 in the serum from patients with OA before and after

Fig. 1

Levels of MMP-1, MMP-9 and MMP-13 measured in the serum of patients with OA and RA before and after synovectomy or TKR (B, before operation; A, after operation).

Fig. 2

Levels of MMP-1, MMP-9 and MMP-13 measured after treatment with 100 ng/ml of SDF-1 on human cultured chondrocytes. They were increased by SDF-1 in a dose-dependent manner.

synovectomy were 230 and 45 ng/ml respectively and from RA patients before and after synovectomy 375 and 56 ng/ml, respectively, representing mean 5.1- and 6.7-fold decreases after synovectomy which were statistically significant ($p = 0.0235$ and $p = 0.0312$, respectively). The concentrations of SDF-1 from patients with OA before and after TKR were 85 and 52 ng/ml, respectively, and from patients with RA undergoing TKR 125 and 75 ng/ml, respectively, representing no significant change after TKR. Statistical analysis indicated that the concentrations of SDF-1 in serum were significantly decreased in patients with OA and RA after synovectomy compared with TKR. Arthroscopy revealed that patients who underwent TKR had less synovium in the knee than those who underwent synovectomy. Therefore, synovectomy can reduce the concentration of SDF-1 in serum.

Levels of MMP-1, MMP-9 and MMP-13 measured before and after synovectomy or TKR. To analyse whether synovectomy is effective in reducing the level of MMP-1, MMP-9 and MMP-13 and thereby minimising destruction of articular cartilage, we measured the level of active MMP-1, active MMP-9 and active MMP-13 in serum before and after synovectomy or TKR. After synovectomy the level of MMP-9 was decreased 6.6- and 10-fold in patients with OA and RA and that of MMP-13 decreased 8.8- and 11.7-fold in those with OA and RA, respectively (Fig. 1). Thus synovectomy is also effective in reducing the level of MMPs in serum.

Detection of SDF-1 in synovial tissue by immunofluorescent histology. To confirm whether SDF-1 is expressed in synovial tissue in OA, we performed an immunofluorescent assay using anti-SDF-1 mAb. SDF-1 positivity was detected in lining cells of synovial tissue and in epithelial cells or lymphocytes in blood vessels. Thus SDF-1 was clearly expressed in synovial tissue in articular joints.

Immunohistochemical detection of SDF-1 and its receptor, CXCR4, in cartilage. We found no expression of SDF-1 in

cartilage, but SDF-1 was expressed in bone marrow just under the cartilage. To determine whether the SDF-1 receptor, CXCR4, is expressed in cartilage, we performed an immunohistochemical analysis of the articular cartilage using anti-CXCR4 mAb. Positivity for CXCR4 was detected in hypertrophic chondrocytes in the deep layer of the articular cartilage in patients with OA and also in the bone marrow adjacent to the area of calcified cartilage. These results indicate that SDF-1 from the synovium and the bone marrow can bind CXCR4 on chondrocytes in articular cartilage to transduce signalling in chondrocytes.

The levels of MMP-1, MMP-9 and MMP-13 in chondrocytes or synovial fibroblasts after treatment with SDF-1. We found that not only MMP-13 but also MMP-9 was increased by SDF-1 in a dose-dependent manner (Fig. 2). MMP-1 was not increased by SDF-1. We also measured MMPs in human cultured synovial fibroblasts after treatment with 100 ng/ml of SDF-1. The levels of MMP-1, MMP-9 and MMP-13 were not changed (Fig. 3). We have already reported that the SDF-1 receptor, CXCR4, was not expressed in synovial fibroblasts.⁵ Therefore, SDF-1 from the synovium stimulates the release of MMP-9 and MMP-13 from chondrocytes to facilitate the destruction of matrix in articular cartilage.

Transduction of mutant CXCR4 into chondrocytes using a retrovirus vector. In an attempt to establish a gene therapy for OA and RA by blocking SDF-1/CXCR4 signalling, we transfected mutant CXCR4, which can bind SDF-1 but cannot transduce signalling, into chondrocytes by means of a retrovirus vector system. The chondrocytes which were transfected to produce mutant SDF-1, blocked the release of MMP-9 and MMP-13 by 46% and 51% of the control value, respectively. Overexpression of normal CXCR4 increased the level of MMP-9 and MMP-13 3.1- and 2.1-fold, respectively (Fig. 4).

Fig. 3

Levels of MMP-1, MMP-9 and MMP-13 measured after the treatment with 100 ng/ml of SDF-1 on human cultured synovial fibroblasts. They were not changed by treatment with SDF-1.

Fig. 4

CXCR4 overexpression cells and mutant CXCR4 cells were established in human chondrocytes using a retrovirus vector system. Levels of MMP-1, MMP-9 and MMP-13 were measured by ELISA at seven days after transfection (Cont, non-transfected human chondrocytes; CXCR4, chondrocytes with CXCR4 overexpression; mCXCR4, chondrocytes with mutant CXCR4 (* significantly decreased compared to the control)).

The transfection efficiency was 21% and 24% for mutant CXCR4 and overexpressed CXCR4, respectively, as measured by flowcytometry. This means that although the transfection efficiency was low, we were able to block the release of MMP-9 and MMP-13 from chondrocytes by using mutant CXCR4 with a retrovirus transfection system.

Discussion

Synovectomy is effective in relieving pain in patients with OA and RA who have synovial proliferation in joints.^{10,11} However, there has been no investigation into the efficacy of synovectomy in articular joints. The synovium of patients with OA and RA produces many types of cytokine and chemokine such as IL-1 α , TNF- α , MIP1- α , RANTES^{4,12,13} and many types of MMP.³ These can induce the breakdown of cartilage. We have found that SDF-1 can stimulate release of MMP-9 and MMP-13 from chondrocytes. MMP-9 is a useful marker of inflammation because it correlates with the leukocyte count.¹⁴ However, MMP-13 is related to lymphocytes and chondrocytes,¹⁵ indicating that it is related to the degraded cartilage matrix itself.¹⁶ We detected a decrease in SDF-1, MMP-9 and MMP-13 in serum after synovectomy. This indicated that these molecules are involved in the proliferation of synovium in OA and RA. We observed a dramatic increase in the concentration of SDF-1 in synovial fluid from patients with RA and OA.⁵ Those with RA showed a mean increase of 971% in synovial SDF-1 while those with OA had an increase of 257%.⁵ Gene expression analysis showed that SDF-1 mRNA was synthesised by synovial fibroblasts but not by chondrocytes.³ Furthermore, synovial fibroblasts from patients with RA and OA synthesised higher levels of SDF-1 mRNA and secreted higher

levels of SDF-1 protein than normal adults.⁵ Thus, our data suggest that the stimulated expression of SDF-1 by synovial fibroblasts in the pathogenesis of RA and OA accounts for part of the increase in SDF-1. This suggests a clear link between the elevated level of SDF-1 in the synovium and the development of RA and OA. If we can remove SDF-1 with the synovium by synovectomy the concentration of SDF-1 in serum can be decreased. The levels of MMP-9 and MMP-13 in serum are also decreased which means that C-reactive protein (CRP) will be reduced and the matrix breakdown for OA and RA will be suppressed, since it has been reported that CRP and MMP-9 are correlated with each other through angiogenesis.¹⁴

SDF-1 has the additional function of accumulating CD4 (+) memory T cells in the synovium. This indicates that SDF-1 is related to the immune system and the inflammation which attracts lymphocytes to develop RA.^{17,18} In our study, SDF-1 was expressed in the synovium but not in cartilage. Thus the synovium plays an important role in producing SDF-1.

For possible use in a gene therapy for OA or RA, we have developed a mutant SDF-1 which can suppress release of MMP-9 and MMP-13 from chondrocytes. However, the transfection efficiency was relatively low at around 24.3% as measured by flowcytometry. In future studies we will need to improve the efficiency and reduce the side-effects of the virus.

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