CONCISE REPORT

S100A4 is expressed at site of invasion in rheumatoid arthritis synovium and modulates production of matrix metalloproteinases

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The metastasis-associated protein S100A4 promotes the progression of cancer by regulating the remodelling of the extracellular matrix. The expression of S100A4 *in vivo* is shown and the functional role of S100A4 in the pathogenesis of osteoarthritis and rheumatoid arthritis is explored. The expression of S100A4 in rheumatoid arthritis, osteoarthritis and normal synovial tissues was determined by immunohistochemistry. The expression of matrix metalloproteinase (MMP) mRNA was measured in rheumatoid arthritis and osteoarthritis synovial fibroblasts treated and untreated with S100A4 oligomer by real-time polymerase chain reaction. Levels of released MMPs were confirmed by ELISA in cell culture supernatants. S100A4 protein was expressed in rheumatoid arthritis and osteoarthritis synovial tissues, in contrast with normal synovium. S100A4 up regulated MMP-3 mRNA in rheumatoid arthritis synovial fluid, with a peak after 6 h. This resulted in release of MMP-3 protein. MMP-1, MMP-9 and MMP-13 mRNA were also up regulated in synovial fluid, but with different kinetics. MMP-14 mRNA showed no change. Thus, S100A4 protein is expressed in synovial tissues of patients with rheumatoid arthritis and osteoarthritis in contrast with healthy people. It induces the expression and release of MMP-3 and other MMPs from synovial fluid. The data suggest that S100A4-producing cells could be involved in the pathogenesis of osteoarthritis and rheumatoid arthritis, including pannus formation and joint destruction.

S100A4 (also known as metastasin, pEL, p9Ka, FSP, CAPL) is a member of the S100 calcium-binding protein family. It comprises ≥20 members, most of them being synthesised and localised intracellularly and exerting regulatory roles in the cytoplasm, such as cell proliferation and differentiation, apoptosis, signal transduction and cell motility.¹ Some of the S100 proteins can be secreted from different cell types, and once released, exert autocrine and paracrine functions. S100A4 was originally isolated from metastatic tumour cells, and the association between metastasis and overexpression of S100A4 was subsequently shown in transgenic mice,^{2,3} and in different human tumours.^{4–6} The invasive-promoting effect of S100A4 during metastasis is related to its action on a variety of intracellular targets, including proteins involved in cell motility, adhesion, detachment, proliferation and apoptosis, as well as on angiogenesis and remodelling of the extracellular matrix.^{6,7}

Different cell types including activated fibroblasts, macrophages, leucocytes and endothelial cells may express S100A4.⁸ It is secreted and can be localised to the extracellular space.^{9,10} It has been shown that extracellular

S100A4, particularly its oligomeric conformational form, down regulates the angiogenesis inhibitor thrombospondin-1, thus stimulating neo-angiogenesis.¹⁰ In addition, it promotes the production of matrix metalloproteinases (MMPs) from endothelial and tumour cells.¹¹ These data show that S100A4 plays an important part in the interaction of tumours with the associated extracellular matrix.

Rheumatoid arthritis is a disease characterised by destruction of articular cartilage and subchondral bone. Joint destruction is mediated by a hyperplastic synovial tissue containing activated synovial fibroblasts and inflammatory cells—that is, macrophages and lymphocytes. Increased production of proteolytic enzymes, particularly MMPs, by rheumatoid arthritis synovial fibroblasts (RA-SFs) is considered to be a hallmark of the disease.¹²

On the basis of the fact shown earlier, by subtractive hybridisation, an increased production of S100A4 in proliferating RA-SFs,¹³ we searched for expression and localisation of this protein in rheumatoid arthritis synovial tissues, in particular, at sites of bone invasion, and explored whether its deleterious effect could be mediated by an increased production of MMPs.

MATERIALS AND METHODS

Synovial tissue samples were obtained from patients with rheumatoid arthritis and osteoarthritis at the time of joint surgery (Clinic of Orthopaedic Surgery, Schultess Hospital, Zurich, Switzerland). All patients fulfilled the American College of Rheumatology criteria for the diagnosis of rheumatoid arthritis or osteoarthritis. Control synovial tissues were obtained from patients with trauma without previous joint disorders. Fibroblasts from passages 4–8 were used. The study was approved by the local ethics committee.

An active oligomeric fraction of S100A4 was obtained from recombinant His6-tagged protein by gel filtration.¹⁴ Semiconfluent synovial fibroblasts were washed with phosphate-buffered saline and treated with S100A4 oligomer. Dose-dependent experiments (0–4 µg/ml S100A4) and time courses (0, 6, 12 and 24 h) were performed. The doses and time courses were according to previous experiments.^{11,14} Cells were collected and mRNA isolated at each time point. Cell culture supernatants were collected after 24 h of exposure to S100A4.

S100A4 protein was detected by immunohistochemistry on paraffin-wax-embedded sections of synovial tissues from patients with rheumatoid arthritis, osteoarthritis and controls. Anti-S100A4 mouse monoclonal antibody¹⁵ was used. Anti-mouse biotinylated antibodies, streptavidin conjugated to alkaline phosphatase and Dako fast red substrate showed

Abbreviations: MMP, matrix metalloproteinase; OA-SF, osteoarthritis synovial fibroblast; RA-SF, rheumatoid arthritis synovial fibroblast

the staining. As a negative control, murine immunoglobulin G replaced the primary antibodies.

Total RNA from synovial fibroblasts was isolated using the RNeasy kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions. Complementary DNA was obtained by reverse transcription (using MultiScribe Reverse Transcriptase and random hexamers; Applied Biosystems). Polymerase chain reaction was performed using a standard protocol. Predeveloped primers (Applied Biosystems, Foster City, California, USA) were used to detect MMP-1, MMP-3, MMP-9, MMP-13 and MMP-14 mRNA. 18S Ribosomal RNA was used for correcting the results with the comparative threshold cycle (Ct) method for relative quantification, and the Ct of 18S was subtracted from the MMP Ct, giving the Δ Ct values.

The levels of MMP-1 and MMP-3 were measured in the cell culture supernatant using MMP Human Biotrak Assays according to the manufacturer's protocols (GE Healthcare Europe, Otelfingen, Switzerland). The protein levels were normalised to the cell numbers (between 2×10^5 and 10^6 in a T75 culture flask).

All the values are expressed as median values. Wilcoxon's non-parametric test was used for related samples. The Bonferroni correction, a statistical adjustment for multiple comparisons, was performed.

RESULTS

S100A4 was detected in 10/10 rheumatoid arthritis synovial specimens. It was localised in the synovial lining and mostly in the sublining layer (fig 1A). It was also found extracellularly in rheumatoid arthritis. Most importantly, the expression of S100A4 was detected at sites of bone invasion in 4/4 rheumatoid arthritis synovial specimens (fig 1B).

Synovial tissues from patients with osteoarthritis (5/7) showed a staining pattern similar to that of patients with rheumatoid arthritis (fig. 1C). However, with regard to lower cellularity, the density of S100A4 was less in the osteoarthritis synovial tissue. No or minimal staining around vessels was found in control synovial tissues from patients with trauma (3/3) (fig 1D).

According to morphological characteristics of the cells and serial labelling with CD68, synovial fibroblasts, macrophages and certain vascular endothelial cells appeared to produce S100A4 (fig 2).

A dose-dependent induction of MMP-1, MMP-3, MMP-9 and MMP-13 mRNA was observed in RA-SFs within 6 h (fig 3). The response decreased thereafter. On the other hand, no induction of MMP-14 mRNA was detected. Similar data were observed when osteoarthritis synovial fibroblasts (OA-SFs) were treated with S100A4 (data not shown).

The expression of MMP-3 mRNA in RA-SFs treated with 0.5 μ g/ml S100A4 was considerably up regulated at all time points, with a peak after 6 h. MMP-1, MMP-9 and MMP-13 mRNA were also up regulated in RA-SFs, but with different kinetics. Although MMP-9 mRNA was increased at all time points (2.3–2.9-fold increases), MMP-1 mRNA was considerably up regulated (2.7–2.8-fold) after 6 and 12 h, and MMP-13 mRNA peaked after 6 h (2.6-fold). A significant correlation ($r = 0.43$, $p < 0.05$, Spearman's rank correlation) was found between the increased expression of MMP-1 and MMP-3 mRNAs.

The induction of MMP mRNA was confirmed at the protein level. Thus, MMP-1 and MMP-3 protein expression increased (by 160–180%, $p < 0.05$) within 24 h in cell culture supernatants from RA-SFs exposed to S100A4 (fig 4). Similar observations were made in OA-SFs. The levels of MMP mRNA were generally lower in OA-SFs than in RA-SFs, but the degree of up regulation was similar.

DISCUSSION

We report the intracellular expression and extracellular localisation of S100A4 in rheumatoid arthritis synovial tissues, particularly at sites of bone invasion. The S100A4 protein has been observed in several osteoarthritis synovial tissues, suggesting a role in its pathology. Owing to lower cellularity, S100A4 was more sparsely distributed in osteoarthritis compared with rheumatoid arthritis synovial tissues. Furthermore, only in rheumatoid arthritis was S100A4 detected in the extracellular space. High levels of synovial fluid S100A4 were detected in rheumatoid arthritis (data not shown). Thus, it is tempting to speculate that S100A4 has an

Figure 1 Immunohistochemical staining for S100A4 in the synovial tissues from rheumatoid arthritis lining and sublining layer (A), and site of bone destruction (B), osteoarthritis sub-lining layer (C), and trauma sample (D). Mouse immunoglobulin G was used as a control (A', B', C', D'). Original magnification $\times 400$.

Figure 2 Immunohistochemical staining for CD68 (macrophages) and S100A4 in synovial tissues from the lining (A) and sublining layer (B) of patients with rheumatoid arthritis. Original magnification $\times 400$.

Figure 3 Dose-dependent increases in the expression of matrix metalloproteinase (MMP)-1, MMP-3, MMP-9 and MMP-13 mRNA in rheumatoid arthritis synovial fibroblasts exposed to the S100A4 oligomer. MMP mRNAs were quantified with real-time polymerase chain reaction. Horizontal bar within the box represents the median, and the boxes represent a range of $\pm 25\%$ around the median. Vertical bars indicate 95% confidence interval. Wilcoxon's non-parametric test was used for related samples, including the Bonferroni correction for statistical adjustment for the multiple comparisons.

Figure 4 Levels of matrix metalloproteinase (MMP)-1 and MMP-3 in the cell culture supernatant, 24 h after stimulation with S100A4, as measured by ELISA and normalised for the number of cells in the culture flask. Wilcoxon's non-parametric test was used for related samples.

autocrine or paracrine role in rheumatoid arthritis. Extracellular S100A4 increases the expression and production of MMPs in synovial fibroblasts, suggesting a role in the process of joint destruction. There is no difference in MMP induction by S100A4 in OA-SF versus RA-SF; the difference between the two diseases seems to be that S100A4 is secreted in rheumatoid arthritis and found in the extracellular compartment.

Matrix-degrading enzymes, particularly MMPs, contribute considerably to joint destruction in rheumatoid arthritis. As S100A4 is localised in both intracellular and extracellular compartments, a role for secreted S100A4 can be postulated. We found that exogenous S100A4 oligomer triggers the production of MMPs in synovial fibroblasts, as is the case in endothelial and tumour cells.¹³ Thus, the invasive behaviour of synovial fibroblasts with increased proteolytic activity resembles the metastatic activity of tumour cells in the presence of S100A4.^{7, 11-13}

In conclusion, this study provides evidence of the expression of S100A4 in synovial tissues from patients with rheumatoid arthritis and osteoarthritis, which was not observed in controls. In rheumatoid arthritis, S100A4 was present at sites of joint destruction, and also in the extracellular compartment. As the exogenous S100A4 oligomer interacts with synovial fibroblasts and increases the expression and production of MMPs, our data suggest that S100A4 has an autocrine or paracrine role in the invasive process in rheumatoid arthritis.

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