Discrepancy between mRNA and protein expression of tumour suppressor maspin in synovial tissue may contribute to synovial hyperplasia in rheumatoid arthritis


Objective: To investigate the expression of maspin in RA synovial tissue and compare it with the expression in osteoarthritis (OA) and normal synovial tissue (NS).

Methods: Using specific primers for maspin, a 237 bp fragment was amplified from cDNA obtained from cultured RA, OA, and normal synovial fibroblasts (SF) by RT-PCR. Additionally, mRNA expression levels were determined quantitatively by real time PCR. mRNA expression of maspin was investigated on snap frozen and paraffin embedded synovial tissue sections by in situ hybridisation. Immunohistochemistry was used to identify the cell type expressing maspin. SDS-PAGE and western blotting were performed to evaluate the protein expression in cultured SF. To confirm protein synthesis in situ, immunohistochemistry with specific anti-maspin antibodies was performed in synovial tissue sections of patients with RA.

Results: RT-PCR showed expression of maspin in all cDNA samples from cultured SF. Maspin mRNA was found to be decreased in RA SF twofold and 70-fold compared with OA SF and NS SF, respectively. Maspin mRNA was expressed in RA, OA, and normal synovial tissue. Importantly, maspin transcripts were also found at sites of invasion into cartilage and bone. At the protein level, maspin could be detected in RA and, less prominently, OA SF. In RA synovial tissue, maspin protein was detected in only a few synovial lining cells.

Conclusion: Maspin is expressed intensively in RA SF at the mRNA level, but only slightly at the protein level, possibly owing to down regulation of maspin; this may contribute to the hyperplasia of synovial tissue in RA.

Abbreviations: maspin, mammary serine protease inhibitor; NS, normal synovial tissue; PAI, plasminogen activator inhibitor; RA, rheumatoid arthritis; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate-polymerase gel electrophoresis; SF, synovial fibroblasts; uPA, urokinase plasminogen activator.
The specimens were fixed in 4% buffered formalin for 4–12 hours, decalcified in 10% EDTA, and embedded in paraffin.

**Tissue preparation, cell cultures, RNA isolation, and reverse transcriptase-polymerase chain reaction (RT-PCR)**

Synovial tissue samples of seven patients with RA, four patients with OA, and 1 trauma patient were minced mechanically and digested with Dispase II at 37°C. Isolated cells were seeded into tissue culture dishes and cultured at 37°C in an atmosphere of 5% CO₂. After three to four passages, total RNA was isolated from SF according to the TRIzol protocol. Reverse transcription into cDNA was performed using oligo-d(T)₁₂₋₁₈ primers and M-MuLV reverse transcriptase (Boehringer Mannheim, Mannheim, Germany). A 237 bp maspin-specific cDNA fragment (Gene Bank accession number U04313) was amplified by PCR with Taq polymerase (Gibco Life Technology, Basel, Switzerland). The primer sequences were as follows: upper primer 5’-GGC CT ATC AAA TGT TAT CCA C-3’, lower primer: 5’-GAC TTT CTG GAT GAT GCC G-3’. PCR was carried out as a three phase reaction for 36 cycles under the following conditions: denaturation at 94°C for 30 seconds, annealing at 95°C for 1 minute, and extension at 72°C for 1 minute. PCR was performed using a DNA thermocycler 480 (Perkin-Elmer Applied Biosystems, Rotkreuz, Switzerland). The amplified cDNA fragment was separated in a 1% agarose gel and visualised with ethidium bromide fluorescence.

**Quantitative mRNA measurement using real time PCR**

Reverse transcription was performed using random hexamers and MultiScribe reverse transcriptase (PE Applied Biosystems) using the following conditions: polymerase activation at 95°C for 10 minutes, denaturation at 95°C for 15 seconds, and annealing at 60°C for 1 minute. The expression level of maspin mRNA was determined using a fluorogenic 5’ nuclease assay according to the TaqMan assay conditions (PE Applied Biosystems). The TaqMan primers and probe sequences were as follows: upper primer 5’-TCT TTG GCA AAT TCT GTT CTC CTI-3’, lower primer 5’-TTT CTGTGG ATG CCG ATT TCT-3’, probe 5’-TGG CAT AGC CCA TGT TAA GTC CTC CCT G-3’. PCR was performed on an ABI Prism 7700 Sequence Detection system (PE Applied Biosystems). Maspin expression was determined as -fold increase or decrease relative to S18 expression as internal standard, and is given as a “Ct” value; the Ct value delineates the “threshold cycle”, which indicates the critical cycle of PCR amplification of the respective mRNA. A lower Ct value, thus, indicates a higher mRNA expression of this gene.

**Cloning, transformation, and generation of riboprobes**

The PCR product was ligated into the pPCR-Script Amp SK(+) vector according to the manufacturer’s protocol. After transformation of the vector into E.coli XL-1 Blue MRF’ Kan ultracompentent cells, positive colonies were selected and large scale plasmid preparation performed using the Qiagen MidiPrep kit. The correct base sequence of the cloned cDNA fragment was confirmed by commercial sequencing. The plasmids were linearised, and antisense and sense probes were generated by in vitro transcription using T3 and T7 RNA polymerases. For in situ hybridisation, probes were labelled with digoxigenin-UTP.

**In situ hybridisation**

After deparaffinisation and prehybridisation, paraffin embedded tissue sections were incubated with the digoxigenin labelled RNA antisense probe overnight at 52°C; a sense probe was used as negative control. Unbound probe was digested at 37°C with 10 µg/ml RNase A followed by increasing stringency washing steps with formamide, saline-sodium citrate, and sodium dodecyl sulphate (SDS). Hybridised probes were detected using anti-digoxigenin Fab fragments linked to alkaline phosphatase. For colour development, the sections were incubated with 5-bromo-4-chloro-3-indolyl-phosphate/4-nitro blue tetrazolium chloride colour substrate solution and stopped with Tris-HCl (pH 7.6).

**Immunohistochemistry**

To further characterise the cell type expressing maspin, immunohistochemical double labelling with mouse anti-CD68 (macrophage marker) and mouse anti-CD3 (T cell marker) antibodies was performed on serial tissue sections. After blocking with 4% milk/2% horse serum, tissue sections were incubated with the monoclonal anti-CD68 (clone PG-M1; DAKO, Zug, Switzerland), monoclonal anti-CD3 (clone PC3/188A; DAKO), or isotype-specific IgG control antibodies (dilution 1:50 in Tris-HCl/2% milk) for 30 minutes at room temperature. After washing in Tris-HCl (pH 7.6), the slides were incubated with goat antimouse IgGs (Jackson ImmunoResearch, Roche, Switzerland) diluted 1:200 in Tris-HCl (pH 7.6), followed by incubation with a mouse APAAP complex (alkaline phosphatase anti-alkaline phosphatase; DAKO; dilution 1:50). Detection of positive labelled
cells was performed using the fast blue colour development. In brief, 20 mg naphthol-AS-phosphate (Fluka, Zug, Switzerland) and 40 mg fast blue BB salt (Sigma, Zug, Switzerland) were dissolved in 300 ml N,N-dimethylformamide, each. Both solutions were dissolved in 40 ml 2-amino-2-methyl-1,3-propandiol (Merck, Darmstadt, Germany) with addition of 10 ml levamisole. After mixing and pH adjustment to 8.7, the substrate solution was filtered, and the slides incubated therein for 5–20 minutes. Colour development was stopped in Tris-HCl (pH 7.6).

In addition, immunohistochemistry was performed in synovial tissue samples from four patients with RA using polyclonal goat anti-maspin antibodies (clone sc-8542; Santa Cruz Biotechnology, Heidelberg, Germany) as outlined above.

Protein isolation
Protein from cultured SF (third to fourth passage) of seven patients with RA, four with OA, and one trauma patient were isolated according to the TRizol protocol. As control, healthy breast tissue (n = 1) was used. Briefly, after isolation of RNA in the aqueous phase and precipitation of DNA with ethanol, protein was precipitated with isopropl alcohol and washed three times in 0.3 M guanidine hydrochloride/95% ethanol.

Figure 3  In situ hybridisation on paraffin embedded synovial tissue sections showed maspin mRNA expression in RA, OA, and normal synovial tissue. Whereas the expression of maspin was restricted to SF in lining and sublining in normal and OA synovial tissue (B and C), in RA, maspin mRNA was additionally seen within perivascular infiltrates (D). Prostate tissue was used as positive control showing staining in epithelial cells (A). In situ hybridisation using the sense probe remained negative (negative control; E). Higher magnification disclosed maspin expression in mononuclear cells around vessels (F) as well as in multinucleated cells resembling osteoclasts at sites of invasion of the synovial tissue into cartilage and bone (G). Cultured RA SF on chamber slides also showed a positive signal for maspin (H). Original magnification ×100 (E), ×200 (B, C, D), ×400 (A, F), ×630 (G, H).
After the final wash in 100% ethanol, the protein pellet was vacuum dried for 5 minutes and resuspended in 1% SDS and proteinase inhibitors (10 mM EDTA, 5 mM N-ethylmaleimide, 2 mM phenylmethylsulphonyl fluoride). Protein concentration was measured using the BCA protein assay (Pierce, Illinois, USA).

**SDS-PAGE and western blot**

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the protocol of Laemmli. Briefly, 15 μg of protein of each sample was loaded on 10% polyacrylamide mini-gels, run under non-reducing conditions, and subsequently used for western blotting. Samples and standards were transferred to nitrocellulose by electrophoresis. After checking for correct protein transfer by Ponceau S solution (Serva, Heidelberg, Germany), the membrane was blocked using 5% non-fat dried milk/0.05% Tween 20/1X Tris buffered saline. Maspin antigen retrieval was performed using a mouse monoclonal antihuman maspin antibody (PharMingen, Luzerne, Germany).

Figure 4  To further characterise the cell type expressing maspin, double labelling with anti-CD68 (A, C) and anti-CD3 antibodies (B, D) was performed after in situ hybridisation with maspin probes on paraffin embedded RA synovial tissue samples. Predominantly, maspin was detected in CD68 and CD3 negative SF. Blue-black colour: maspin transcripts, red-brown colour: anti-CD68 and anti-CD3 positive cells, respectively. Original magnification×200 (A, C), ×400 (B, D).

Figure 5  Maspin mRNA expression was additionally investigated in RA synovial tissue at sites of cartilage and bone destruction. Maspin positive cells with both fibroblast-like (A) and macrophage-like as well as osteoclast-like morphology (C) were found. Double labelling with anti-CD68 antibodies showed both maspin positive, CD68 negative (figs 5B and 3G) as well as maspin positive, CD68 positive cells (D). Original magnification of all figures ×400.
Switzerland) and a secondary horseradish peroxidase-conjugated antimouse IgG (Jackson ImmunoResearch) by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

RESULTS

Maspin expression at the mRNA level using RT-PCR

RT-PCR disclosed expression of maspin in all cDNA samples from cultured RA, OA, and normal SF (fig 1). Real time PCR showed that maspin mRNA expression was decreased twofold in RA SF as compared with OA SF and 70-fold in comparison with NS SF (fig 2).

Maspin expression at the mRNA level using in situ hybridisation

In situ hybridisation showed that maspin mRNA was expressed in RA, OA, and normal synovial tissue (fig 3). Prostate tissue was used as positive control (fig 3A), and maspin sense probe as negative control (fig 3E). In contrast with OA and normal samples, in which expression of maspin mRNA was restricted to SF in the synovial lining and sublining layers (figs 3B and C), in RA samples (figs 3D and H), maspin could additionally be observed within perivascular infiltrates in mononuclear (fig 3F) and multinucleated cells (fig 3G).

Maspin expression at the protein level using SDS-PAGE/western blotting and immunohistochemistry

At the protein level, maspin could be detected in RA SF and, less prominently, in OA SF by western blotting. In a sample of normal SF, maspin could scarcely be detected (fig 6A; see also table 1). Normal breast tissue was used as positive control (results not shown). To evaluate maspin protein expression in the synovial tissue of patients with RA, immunohistochemistry using specific anti-maspin antibodies was performed. Maspin was found only in single cells within the synovial lining and sublining (fig 6B).

DISCUSSION

RA synovial tissue is characterised by tumour-like features such as invasive growth of the hyperplastic synovial tissue into cartilage and bone, consecutive tissue destruction, up regulation of proto-oncogenes and down regulation of tumour suppressor genes.10 Within the RA synovium, fibroblasts show an activated phenotype consisting of up regulation of adhesion molecules that mediate the attachment of RA SF to the extracellular matrix, and overexpression of matrix degrading enzymes that mediate the progressive destruction of the joints.4 In line with this finding, the tumour suppressor PTEN has been shown to be down regulated in RA SF, especially at sites of invasion.5 Similarly, maspin is expressed in normal mammary epithelial cells but down regulated or absent in most mammary carcinoma cell lines; in advanced cancers, it is therefore also regarded as a prototype tumour suppressor.11 In addition, maspin acts as inhibitor of angiogenesis by inducing apoptosis in endothelial cells.12

In this study, using highly sensitive in situ hybridisation, we could show for the first time that maspin is expressed in SF as well as in synovial tissue in RA, OA, and normal samples at the mRNA level. Notably, in RA synovium, maspin mRNA expression could also be detected within perivascular infiltrates, and especially at sites of synovial tissue infiltration into cartilage and bone.

Interestingly, when maspin expression levels were examined quantitatively by real time PCR, a twofold lower expression was seen in cultured RA SF than in OA SF and a 70-fold lower expression than in normal SF. As RA SF are known to exhibit an activated phenotype based— in part—on an altered tumour suppressor gene expression,4 the observed reduced expression of maspin in RA SF in comparison with OA SF and normal SF might therefore reflect a down regulation, similar to that seen in malignant diseases, and contribute to the aggressive phenotype observed in RA synovium. This interpretation would not necessarily contrast with the locally high maspin expression in RA SF at sites of invasion because real time PCR results were measured in RA SF obtained from total synovial tissue samples not consisting completely of cells derived from the invasion zone between synovial tissue and cartilage.
Most interestingly, at the protein level, maspin could be detected in only a few single cells in the rheumatoid synovial lining. This contrast between detectable mRNA expression in tissue and a low protein expression probably indicates a posttranscriptional dysregulation, or conformational protein alterations due to unknown mutations. This hypothesis is supported further by a recent report discussing a transcriptional regulation of maspin. The potential role of maspin in RA pathogenesis may be explained by a number of mechanisms known from other diseases that may also be operative in RA synovium:

1. Maspin has been shown to exhibit inhibitory effects on angiogenesis, tumour invasion, and metastasis. Furthermore, it has been shown to induce apoptosis of endothelial cells through distinct signalling pathways. As RA is characterised by hyperplasia of the synovial tissue, angiogenesis, and a reduced rate of Fas/Fas ligand dependent apoptosis, a decreased expression of maspin protein in RA SF and in synovial tissue might contribute to this effect.

2. Treatment of human breast and prostate cancer cells with recombinant maspin protein inhibits cell motility. Moreover, recombinant maspin has been shown to induce higher cell surface levels of α5 and α3 integrins, and to reduce levels of α2, α4, α6, and αv and some β1 integrins of a human metastatic breast carcinoma cell line, which resulted in selective adhesion to a fibronectin-containing matrix. Furthermore, the ability of recombinant maspin to inhibit the invasive process of these cells could be abrogated with a blocking antibody to the α5β1 integrin, which diminished the ability of cells to invade through a fibronectin matrix-containing barrier in vitro, supporting the idea that maspin alters the integrin profile rendering cells more invasive, but less invasive. As increased expression of integrins containing α5 and β1 has been described in RA synovial tissue, and as RA SF could be shown to be less adhesive to collagen and other extracellular matrix components than OA SF, these properties of RA SF might be due to the down regulation of maspin seen in the present study.

3. Recent evidence suggests that maspin expression is—at least in part—regulated by p53 as p53 activates the maspin promoter by binding directly to the p53 consensus binding site present in the maspin promoter. Strong induction of maspin expression was seen in both prostate and breast cancer cells after induction of wild-type p53 by adenoviral gene transfer, indicating that maspin and p53 cooperate in the negative regulation of tumour cell invasion and metastasis. In RA, p53 expression is discussed controversially, but seems to be rather low, and only a limited number of mutations within the p53 gene have been described. Both of the latter reasons might be an explanation for the decreased maspin expression in synovial tissue in vivo as found in our in vitro study.

4. In RA, the urokinase-type plasminogen activator system is critically involved in the cell invasion process into cartilage and bone, operating both directly and indirectly through matrix metalloproteinases. In the RA synovial lining cells, both urokinase plasminogen activator (uPA) antigen and uPA activity were found. Even though it has been reported that maspin does not interact with uPA but instead only with single chain plasminogen activator, recent evidence suggests that maspin, indeed, does exert an inhibitory effect on the uPA system, as it could be demonstrated that expression of maspin in transfected prostate cells reduced the release of active uPA and significantly reduced the ability of maspin to convert plasminogen to plasmin. The reduced maspin expression in RA might therefore also account for the high uPA expression and the activated phenotype of RA synovial lining cells.

In addition, maspin and plasminogen activator inhibitor (PAI)-1 share structural similarities. PAI-2 and, to a lesser extent PAI-1, have been shown to be increased in RA synovial fluid. PAI-1 has an ambiguous function in vivo in RA: (a) a beneficial effect by blocking the plasmin mediated matrix degradation; (b) a deleterious effect by favouring fibrin accumulation and, thus, perpetuating inflammation. Despite enhanced levels of PAI-1 in RA, there is continuing plasmin mediated fibrinolysis. In view of increased uPA/PA levels, the enhanced PAI-1 expression might not be sufficient to counterbalance fibrinolysis. Hypothetically, paralleling the functions of PAI-1 and maspin, the accentuated expression of maspin at the mRNA level at sites of invasion might constitute an insufficient attempt to compensate for pro-inflammatory and matrix degrading agonists. Because the exact in vivo effects on fibrinogenesis, inflammation, or matrix degradation are not known, it is the more difficult to delineate the effect of maspin on angiogenesis, a network whose regulation seems to be very complex, as has been published recently.

5. In RA synovial tissue, the decreased expression of maspin might correlate inversely with, or be the cause of, tissue inflammation/hyperplasia owing to a posttranscriptional dysregulation. The relatively high maspin mRNA expression at sites of invasion might, in turn, constitute an insufficient attempt to compensate for the predominating tissue destructive mechanisms.

A restriction to the (functional) impact maspin might exert in RA is the small number of RA, OA, and normal synovial tissue samples examined in this study. In addition, samples from patients with early RA were not available. However, the

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**Table 1** In situ hybridisation using maspin-specific probes, and immunohistochemical double labelling using anti-CD3 and anti-CD68 antibodies was performed on synovial tissue sections.* Western blotting using specific anti-maspin antibodies and protein extracts from cultured SF.†

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<thead>
<tr>
<th>Maspin expression</th>
<th>Rheumatoid arthritis</th>
<th>Osteoarthritis</th>
<th>Normal synovial tissue</th>
<th>Positive control</th>
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<tbody>
<tr>
<td>Tissue expression (mRNA, in situ hybridisation)</td>
<td>+</td>
<td>+</td>
<td>+ (normal prostate tissue)</td>
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<tr>
<td>Tissue localisation (mRNA, in situ hybridisation)</td>
<td>Lining/sublining, perivascular invasion site</td>
<td>Lining/sublining</td>
<td>Lining/sublining</td>
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<tr>
<td>Cell type at invasion site (double labelling in situ hybridisation, immunohistochemistry)</td>
<td>CD3/CD68 negative SF; mono-/multinucleated cells</td>
<td>CD68 positive/CD68 negative cells</td>
<td></td>
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</tr>
<tr>
<td>Cultured synovial cells (mRNA, in situ hybridisation)</td>
<td>+</td>
<td>+</td>
<td>+ (positive control)</td>
<td></td>
</tr>
<tr>
<td>Cultured synovial cells (protein, western blot)</td>
<td>+</td>
<td>+</td>
<td>+ (positive control)</td>
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*Eight patients with RA, two patients with OA, one normal control, and healthy prostate tissue were studied; †seven patients with RA, four patients with OA, one normal synovial tissue sample, and one healthy breast tissue (control) were used.

ND, not done.
primary aim of this study was to evaluate whether maspin is expressed in RA synovium in general.

In conclusion, because the expression of maspin in RA is reduced at the mRNA level as compared with OA, and especially with normal synovium, and is decreased in RA SF at the protein level, it can be proposed that a down regulation of maspin might contribute to hyperplasia and growth of the synovial tissue and potentially to increased invasivity of synovial cells into cartilage and bone in RA. The expression of maspin mRNA in RA SF and at sites of invasion may be interpreted as an (insufficient) attempt of the organism to counterbalance the lack of maspin protein in these cells.

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References

Maspin in rheumatoid arthritis