Expression and localisation of the new metalloproteinase inhibitor RECK (reversion inducing cysteine-rich protein with Kazal motifs) in inflamed synovial membranes of patients with rheumatoid arthritis


Objective: To assess the expression and localisation of the new metalloproteinase inhibitor RECK, an inhibitor of matrix metalloproteinase-14 (MMP-14) secretion and activity, in the synovial membrane of patients with rheumatoid arthritis (RA).

Methods: RECK expression in synovium samples from patients with RA, osteoarthritis (OA), and “trauma” were studied by quantitative real time reverse transcription-polymerase chain reaction (Q-PCR). RECK mRNA levels were compared with those of the enzyme MMP-14. RECK expression on cryostat sections of synovium was disclosed by goat-antihuman RECK monoclonal antibody. RECK protein was detected on synovial cryostat sections and measured by western blotting. RECK expression on macrophages was investigated by double staining of CD68 and RECK on cryostat sections and characterised by confocal microscopy. RECK expression on RA monocytes or normal monocytes was further investigated by FACS analysis.

Results: RECK expression in the synovial membrane of patients with RA was significantly lower than in OA and controls. MMP-14 mRNA levels were not significantly different between the three groups. In RA synovium, RECK protein was expressed mainly in the lining layer but also by macrophages around blood vessels. Fibroblasts and about 50% of the CD68 positive macrophages expressed RECK. In CD68 positive macrophages, RECK was only expressed in secretory granules and not on the membrane. The same pattern was found in M-CSF cultured macrophages of patients with RA and controls. In contrast, synovial fibroblasts showed a diffuse membrane expression within the synovium similar to cultured RA fibroblasts. RECK expression was low on the membrane of monocytes according to FACS analysis.

Conclusion: The new MMP inhibitor RECK is expressed in synovial membranes of RA, OA, and controls. RECK mRNA is lowest in RA synovial membranes. In contrast with fibroblasts, macrophages in the synovium express RECK only cytoplasmically and not on their membrane.
repressing angiogenesis. Homozygous loss of RECK in mice results in embryonic lethality and causes severe disruption of mesenchymal tissues and organogenesis, which emphasises the importance of RECK. Apart from studies related to oncology, no current information is available on its role in chronic destructive inflammation.

As RECK is described as potent inhibitor of various MMPs involved in RA pathology, we investigated whether RECK is expressed in the inflamed synovium of patients with RA. Comparisons were made with the expression in synovium samples from controls and patients with osteoarthritis (OA), and the cell type responsible for RECK production within the synovial membrane was characterised.

PATIENTS AND METHODS

Patients and samples

Synovium samples were collected from 10 patients with RA who were undergoing total joint replacement. All patients fulfilled the American College of Rheumatology criteria for RA and gave informed consent for the study. All patients were treated with disease modifying antirheumatic drugs alone or in combination with non-steroidal anti-inflammatory drugs. Excluded from the study were patients who were receiving treatment with systemic steroids or biological agents. For comparison, seven patients with OA and six “trauma” patients were included in the study. OA synovia were collected during joint replacement, whereas well defined synovial specimens were collected from trauma patients by arthroscopy. The medical ethics committee of the University Medical Centre approved the study protocol.

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Tissue specimens were embedded in Tissue Tek OCT (Miles, Elkhart, IN) and snap frozen in liquid nitrogen. Cryostat sections (7 μm) were cut, mounted on superfrost slides, and stored at −70°C until further processing.

Quantitative reverse transcriptase-polymerase chain reaction

Quantitative reverse transcriptase-polymerase chain reaction was done as earlier described. Synovium was snap frozen in liquid nitrogen within 2 minutes after isolation. In addition, synovium specimens were homogenised in a freeze mill and RNA isolated using the RNAeasy mini-kit (Qiagen, Hilden, Germany) with on-column DNase I treatment. The quality of RNA isolated using the RNeasy mini-kit (Qiagen, Hilden, Germany) was checked by examining ribosomal RNA bands. In addition, reverse transcription was done as earlier described. Synovium was snap frozen in liquid nitrogen. Cryostat sections (7 μm) were cut, mounted on superfrost slides, and stored at −70°C until further processing.

PCR

PCR reactions were carried out using Taqman Universal PCR master mix (PE Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands) in a final volume of 25 μl. RECK forward primer (300 nM; 5′–TGG CTG GGT TGG CTT AGG–3′), 900 nM reverse primer (5′–GCA AAC TTT GGA AAT ATC ATT CTT TG–3′), and 200 nM fluorescent minor groove binder probe (5′–FAM–TTC AAT GCA TCT–3′) were used. For MMP-14, 300 nM forward primer (5′–GGA TGG CAA ATT CG), 300 nM reverse primer (5′–AGG GAC GCC TCA TCA AAC AC–3′), and 100 nM fluorescent minor groove binder probe (5′–FAM–CTT CAA AGG AGA CAA GCA TT–3′) were used. Primers were ordered from MWG-Biotech AG (Münchenstein, Switzerland) and the Taqman probe from Applera Europe, Applied Biosystems (Rotkreuz, Switzerland). The assay was designed using the Primer-Express software package version 1.5 (PE Applied Biosystems). β-Actin was amplified using the pre-developed assay reagents Taqman RT-PCR assay from Perkin-Elmer (PE Applied Biosystems). All amplifications, with denaturation at 95°C for 10 minutes, and 40 cycles of 15 seconds at 95°C (melting) and 60 seconds at 60°C (annealing and elongation), were performed on an ABI Prism 7700 sequence detection system (PE Applied Biosystems). Quantification of unknown samples was performed using the breast cancer cell line MDA-MB175 as a calibrator.

Antibodies

Monoclonal antibodies were purchased from various suppliers. Anti-CD68 (EBM 11; mature macrophages), anti-CD14 (TUK4; monocytes), and anti-CD32 antibodies were purchased from DAKO, Carpinteria, USA. Anti-RECK and anti-MMP-14 antibodies were purchased from R&D systems.
Minneapolis, USA. Recombinant RECK and MMP-14 peptides with a published sequence were used for immunisation. Other recombinants that were closely related to these molecules were tested for cross reactivity using a direct enzyme linked immunosorbent assay (ELISA). For RECK monoclonal antibodies these were rhEphrinA5/Fc, rmRANK-L/His, rhTIM-1, rmIL-27, rmKlotho; and for MMP-14, rhMMP-1,2,3,12,13, rhHAI-2, rmHAI-1, rrL-selectin, rzfPDGFAA, rhPTPN-1, rrFABP-1, rrGH, rrprolactin R. No cross reactivity was detected. The antibodies were centrifuged at high speed before use to remove agglutinated IgG, and the supernatant was used for immunolocalisation and FACS studies. On western blotting, RECK antibodies showed only one 110 kDa band. MMP-14 antibodies showed four bands of respectively 63 kDa (proenzyme), 60 kDa (enzyme), 42 kDa, and 39 kDa (degradation products).

**Protein isolation of inflamed synovia**

Tissue samples were homogenised in a freeze mill and lysed on ice for 15 minutes in lysis buffer containing 0.5% NP-40, 10 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM Na3VO4 pH 10.0, and 1X protease inhibitor cocktail (Pharmingen, Woerden, The Netherlands). Insoluble material was removed by centrifugation. Protein concentrations were determined with the BCA protein assay kit (Pierce, Rockford, IL).

**Western blot**

For western blotting, 30 µg of protein synovial cell lysate was run on a 7.5% sodium dodecyl sulphate-polyacrylamide electrophoresis gel. Proteins were electrophoretically transferred onto polyvinylidene difluoride membranes (Hybond-P, Amersham Pharmacia Biotech, Buckinghamshire, UK). Membranes were blocked with Tris buffered saline-Tween/1% bovine serum albumin (Sigma, St Louis, MO)/1% dry milk powder (Campina, Eindhoven, The Netherlands). After incubation with first and secondary antibodies, the membranes were developed with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK). For reprobing, the membranes were stripped in 0.2 M glycine pH 2.5 and 0.05% Tween-20 at 80 ˚C for 20 minutes.

**Immunohistochemical analysis**

Cryostat sections (7 µM) were fixed in 100% cold acetone (10 minutes), washed with phosphate buffered saline and incubated with the first antibody (10 µg/ml) for 60 minutes at 37˚C as described earlier. Subsequently, sections were preincubated with normal horse serum to block aspecific binding and with biotinylated horse antimurine IgG. Slides were developed with the ABC kit (Elite kit, Vector, Burlingame, CA), developed with diaminobenzidine, and counterstained with haematoxylin for 5 minutes. Controls consisted of irrelevant primary isotype-specific IgG1 and IgG2a antibodies (DAKO), and secondary antibodies were omitted. Double staining was performed using goat antimouse antibodies either coupled to Alexa 568 (red) or Alexa 647 (blue) (Molecular Probes, The Netherlands). Synovia were visualised using a confocal microscope.

The percentage of RECK positive CD68 positive macrophages was determined by counting 200 CD60 positive cells in 10 different sections.

**FACS analysis**

Blood was isolated from five patients with RA and five healthy controls. Total sodium citrate treated blood (100 µl) was incubated with 100 µl of the first antibody solution (100 µg/ml) and incubated for 30 minutes on ice to prevent uptake of antibodies. Anti-RECK, anti-MMP-14, anti-CD14,
and isotype-specific control monoclonal antibodies (DAK, Glostrup, Denmark) were used as negative controls. After washing with immunofluorescence buffer (phosphate buffered saline + 1% bovine serum albumin pH 7.4), cells were centrifuged for 5 minutes at 1500 rpm and the fluorescent conjugate (goat antimouse F(ab)2 labelled with FITC) was added. After 30 minutes' incubation at 4 °C, cells were washed and the pellet was resuspended in 500 µl IF buffer. FACS analysis was performed using Coulter Epics XL/XL-MCL (Coulter Electronics Ltd, Mijdrecht, The Netherlands).

Culture of macrophages and synovial fibroblasts from patients with RA and controls
Monocytes were isolated from sodium citrate treated blood from patients with RA and controls using CD14 magnetic cell sorting (Miltenyi Biotec, Auburn, USA). Monocytes (10^6/ml Dulbecco’s modified Eagle’s medium (DMEM)) were cultured with monocyte-colony stimulating factor (M-CSF; 1 ng/ml) for 7 days on Teflon foil and used for

Figure 4  RECK and MMP-14 protein expression in synovial specimens from patients with RA as determined by immunolocalisation. Immunolocalisation using a specific mouse-antihuman RECK was performed on cryostat sections of patients with RA. RECK was mainly expressed within the intimal lining layer (A) and by large cells, probably macrophages, lying around blood vessels (B). MMP-14 was found in corresponding regions (C). As a control, a non-relevant IgG2 antibody was used, which showed no staining (D). Original magnification ×250.

Figure 5  Comparison of RECK and the macrophage marker CD68 in serial cryostat sections of synovium from five patients with RA. Double staining for CD68 and RECK using Alexa 647 labelled anti-CD68 (blue fluorescent) and Alexa 568 labelled anti-RECK (red fluorescent), respectively. Colocalisation of RECK and CD68 was clearly present in around 50% of the macrophage-like type A cells. Original magnification ×400. Ma, macrophage; Fb, fibroblast.

Figure 6  Quantitative mRNA levels of RECK and various MMPs (MMP-2, MMP-9, MMP-14) in monocytes of patients with RA and controls. RECK and MMP values were normalised to β-actin signals. Note that no significant differences were found in RECK and MMPs mRNA levels between RA and controls. Values represent the mean of six different patients with RA and controls. Values were evaluated by the Mann-Whitney U test (significance p<0.05).
immunolocalisation. Fibroblasts were isolated from RA synovia which were cut into small pieces, and incubated for 1 hour with collagenase (0.3 mg/ml DMEM; Worthington, Biochemical Company, Lake Wood, USA) in a Falcon 40 ml tube. Synovial cells were additionally filtered through a Falcon cell strainer, and incubated in chamber slides (Lab Tek, NUNC, Rochester, USA) for 3 days in DMEM/10% fetal calf serum and also used for immunolocalisation.

RESULTS
RECK mRNA expression in RA synovium
To determine the presence of RECK mRNA, synovial membranes of patients with RA and OA and of controls were studied by quantitative real time reverse transcription-polymerase chain reaction (Q-PCR) analysis. Haematoxylin stained sections showed that all RA synovia contained large amounts of inflammatory cells. PCR products were resolved by agarose gel electrophoresis, showing the presence of a 99 bp cDNA product of RECK in a minority of RA synovial membranes (fig 1). In contrast, clear expression of RECK mRNA was found in all control synovia studied. Nucleotide sequencing of the PCR amplification confirmed the identity of RECK. The quality and efficiency of the RNA extraction were controlled for measuring $\beta$-actin. RECK expression in a breast cancer carcinoma cell line was taken as a positive control. Negative controls without template/primers or without template were always negative. Interestingly, Q-PCR showed significantly lower RECK mRNA levels in RA synovial membranes than in those found in synovial membranes of controls ($p = 0.031$; fig 2A). In synovial membranes of patients with OA we found that RECK mRNA levels were highly variable.

As RECK is a potent inhibitor of secretion and activity of MMP-14, we additionally determined mRNA levels of MMP-14 in the same samples as used for RECK determination. Although we found a trend for MMP-14 mRNA levels to be higher in synovial membranes derived from patients with RA than in membranes from OA and controls, these levels were not significantly different (fig 2B). These data suggest that the MMP-14/RECK balance is higher in the RA synovium than in OA, which may result in increased MMP activation.

RECK protein is expressed by the synovial membrane of patients with RA
To further investigate whether RECK protein is expressed, tissue samples which showed detectable levels of RECK mRNA were homogenised and RECK protein was detected by western blotting using an enhanced chemoluminescence system. In RA synovia, a clear band with a molecular weight of 110 kDa was found (fig 3), indicating that the protein is indeed expressed.

In addition, we investigated the topography of the RECK protein within the synovial membrane. For this purpose, cryostat sections of synovial membranes were stained with anti-RECK antibodies. In RA synovium, RECK immunoreactivity was predominantly found within the thickened intimal layer (fig 4A). Virtually all cells throughout this layer expressed RECK. In the subintimal layer, RECK was also

![Figure 7](image_url) Expression of RECK, MMP-14, CD14, and CD32 (FcRII) on monocytes from five patients with RA and five healthy controls as determined by flow cytometric analysis. Monocytes were detected in sodium citrate treated blood. The percentage of positive cells was determined. Data were evaluated by the Mann-Whitney U test ($p < 0.05$). Note that only low levels of cells express RECK and MMP-14. No significant differences in RECK expression on monocytes was found between patients with RA and healthy controls. RA, rheumatoid arthritis; C, control.

![Figure 8](image_url) Immunolocalisation of RECK in RA monocytes treated for 7 days with M-CSF and fibroblasts isolated from RA synovial membranes, and subsequently cultured for 3 days. Confocal microscopy shows that in macrophages, RECK is only expressed intracellularly and not on its membrane [A]. In contrast, synovial fibroblasts showed a dispersed pattern and RECK was clearly present on the membrane [B]. Original magnification $\times 400$. 

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become highly invasive and display a tumour-like character. 

In RA, the thickened intimal layer predominantly comprises fibroblasts and macrophages. As the macrophage is a dominant cell type involved in synovial inflammation and destruction, we further investigated whether RECK was also expressed by these cells. RECK expression up to now has only been described in mesenchymal cells and not in haematopoietic cells. We found that RECK protein is expressed only by a subpopulation of synovial macrophages, as shown by confocal microscopy using double staining with RECK and CD68 antibodies. The two antigens of interest were visualised by an indirect Alexa 568 labelled (red fluorescent) CD68 antibody and an indirect Alexa 647 labelled (blue fluorescent) RECK antibody (fig 5). Around 50% of the CD68 positive macrophages expressed RECK. Interestingly, RECK was only found inside secretory granules and was not expressed on the membrane. In CD68 negative, RECK positive intimal fibroblasts a diffuse staining pattern was found on the membrane of the cell.

As synovial CD68 positive macrophages are thought to stem from infiltrating CD14 positive monocytes, which further mature in the synovial layer into CD68 positive macrophages, we additionally investigated both RA and control monocytes.

Although RECK and MMP-14 mRNA were both expressed in peripheral monocytes of patients with RA and controls, no significant differences in mRNA levels were found between the two groups (fig 6). MMP-2 and MMP-9 mRNA levels, which are also inhibited by RECK, were also found not to differ (fig 6). Using FACS analysis, only very low levels of RECK protein were measured on the membrane of CD14/CD32 positive monocytes of patients with RA. These levels were comparable to the levels found in controls (fig 7). As maturation of the monocyte into CD68 positive macrophages may alter the expression pattern of RECK, we additionally cultured monocytes derived from patients with RA and healthy controls for 7 days in the presence of M-CSF.

Confocal microscopy showed that RECK localisation was only cytoplasmic and again not on the membrane (fig 8A). In contrast, when fibroblasts isolated from RA synovial membranes were cultured for 3 days, a diffuse membrane staining pattern was seen (fig 8B).

**DISCUSSION**

As far as we know, this is the first study to report that RECK, which is a new inhibitor of secretion and activity of metalloproteinases, is expressed in the synovial membrane. RECK has been characterised as a major inhibitor of various MMPs and also MMP-2 and MMP-9 have been shown to be involved in vessel maturation, and inhibition of the release and activation of various MMPs.

One of the characteristics of RA synovial membrane is uncontrolled formation of blood vessels. Synovial fibroblasts derived from patients with RA are main producers of angiogenic factors like angiopoietin-1 and angiogenin. Mouse embryos in which the RECK gene was ablated showed a disorganised mesenchymal tissue architecture and, although vascular networks were formed, vascular endothelial cells did not form tight tubules. Moreover, overexpression of RECK resulted in attenuated tumour formation owing to limited angiogenic sprouting. Our finding that in the sublining RECK is detected around blood vessels suggests that RECK may help in controlling synovial angiogenesis.

Apart from angiogenesis, RECK also plays a part in tumour development and has been shown to be a prognostic marker in human breast carcinoma. In RA, fibroblasts in the lining become highly invasive and display a tumour-like character. This normally thin layer, which comprises fibroblasts and macrophages, is largely thickened during RA and is the main producer of cytokines and enzymes. Both RECK and MMP-14 are predominantly found within the lining layer. Interestingly, RECK mRNA expression was lower in RA than in control synovial membranes. An explanation may be that local inflammation inhibits RECK expression. The RECK promoter binds transcription factors like sp1 and sp3, which are also needed for cytokine signalling. This suggests that these cytokines may be involved in RECK regulation. Whether RA fibroblasts express lower levels of RECK than fibroblasts from control synovia is now the subject of further studies. Using double staining of CD68 and RECK, we further found that about 50% of the CD68 positive macrophages expressed RECK; this was expressed only subcellularly. In contrast, a diffuse pattern of staining was found on the membrane of synovial fibroblasts, indicating that RA fibroblasts do express RECK on their membrane, which is in line with earlier studies of Takahashi et al. This suggests that macrophages are less capable of controlling MMP-14 activation than fibroblasts and is another argument for the important role which macrophages play in mediating synovial proliferation and cartilage destruction. The lack of membrane expression was, however, not RA-specific because the same patterns were found in control monocytes and mature macrophages. One explanation may be that macrophages express a splice variant of RECK which cannot be expressed on its membrane.

RECK post-transcriptionally not only regulates MMP-14 but also at least two other members of the MMP family—namely MMP-2 (gelatinase A) and MMP-9 (gelatinase B). Membrane anchored RECK inhibits secretion of pro-MMP-9, whereas both membrane anchored and soluble RECK directly inhibit MMP-2, MMP-9, and MMP-14 catalytic activity. All three MMPs have been shown to be involved in the activation of latent MMPs, which subsequently drive proliferation of synovial tissue and degradation of cartilage matrix. MMP-14, which can also directly degrade collagen type II, activates latent MMP-13, which is thought to be the rate limiting enzyme that mediates severe cartilage destruction. As macrophages are crucial in the production and activation of MMPs and as these cells are strongly related to induction of severe cartilage destruction, RECK may be an important therapeutic agent to combat local activation of MMPs within RA.

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