EXTENDED REPORT

Analysis of the cell infiltrate and expression of proinflammatory cytokines and matrix metalloproteinases in arthroscopic synovial biopsies: comparison with synovial samples from patients with end stage, destructive rheumatoid arthritis

T J M Smeets, E C Barg, M C Kraan, M D Smith, F C Breedveld, P P Tak

See end of article for authors' affiliations
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Correspondence to:
Dr T J M Smeets, Division of Clinical Immunology and Rheumatology, F4–218, Academic Medical Centre/University of Amsterdam, Meibergdreef 9, 1105 AZ, Amsterdam, The Netherlands;
T.J.Smeets@amc.uva.nl

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Background: Synovial tissue (ST) from end stage destructive rheumatoid arthritis (RA) and arthroscopic biopsies obtained during active inflammation might exhibit different characteristics.
Objective: To define the cell infiltrate and the expression of proinflammatory cytokines, angiogenic factors, and matrix metalloproteinases (MMPs) in ST selected at arthroscopy compared with that from end stage RA.
Methods: Synovial biopsy specimens were obtained from the actively inflamed knee joints of 13 patients with chronic RA by arthroscopy and compared with ST from 10 patients with end stage, destructive RA. Immunohistological analysis was performed to detect T cells, plasma cells, macrophages, fibroblast-like synoviocytes (FLS), and the expression of interleukin (IL)1β, IL6, tumour necrosis factor α (TNFα), MMP-1, MMP-3, MMP-13, TIMP-1, and VEGF.
Results: The expression of CD68+ macrophages was significantly higher in ST selected at arthroscopy than in samples obtained at surgery, both in the intimal lining layer and in the synovial sublining. The expression of CD3+ T cells also tended to be higher in arthroscopic samples. The expression of TNFα, IL6, MMP-1, MMP-3, MMP-13, TIMP-1, and VEGF was on average higher in ST obtained at arthroscopy. In contrast, the expression of IL1β was on average higher in surgical samples.
Conclusion: Active arthritis activity is associated with increased cell infiltration, expression of proinflammatory cytokines, MMPs, and angiogenic growth factors in synovial biopsy samples selected at arthroscopy. Increased expression of IL1β in the synovium of patients with destructive RA requiring joint replacement may well reflect the important role of IL1β in cartilage and bone destruction.

Synovial inflammation and synovial hyperplasia are prominent features of rheumatoid arthritis (RA), and may precede the first clinical signs of the disease.[1][2] Sustained production of proteolytic enzymes by RA synovial tissue (ST), mainly by macrophages and fibroblast-like synoviocytes (FLS), may result in destruction of articular cartilage and bone. In most older studies examining RA ST, samples were obtained from end stage, destructive RA during joint replacement.[3–5] The selection of patients in those studies might bias the features of the ST, because the characteristics of synovial inflammation and local disease activity are associated.[1][6]

Previous studies suggested that ST of end stage, destructive RA could differ from tissue selected at arthroscopy during active inflammation. For example, p53 expression is especially prominent in the intimal lining layer of patients with RA with end stage, destructive disease,[7] whereas p53 expression is more pronounced in the synovial sublining of samples obtained by arthroscopy from patients with RA with active disease.[8] In addition, a recent study demonstrated p53 mutations in FLS from patients with longstanding, destructive RA, but perhaps not in patients with RA in earlier phases of the disease.[9][10] We suggest that in end stage, destructive RA, inflammation is not necessarily a prominent feature and other mechanisms may be more important in this subset of patients.

This study aimed at defining the cell infiltrate and the expression of proinflammatory cytokines, angiogenic factors, and matrix metalloproteinases (MMPs) in ST from patients with active RA, selected at arthroscopy, compared with ST from end stage, destructive RA obtained during joint replacement.

PATIENTS AND METHODS
Patients
Ten consecutive patients with destructive, end stage RA, who underwent joint replacement, and 13 consecutive patients with RA with active arthritis of a knee joint, who underwent arthroscopy, were investigated. Eight patients from the Repatriation General Hospital in Daw Park, South Australia, and 15 patients from the Leiden University Medical Centre were included. All patients fulfilled the American College of Rheumatology criteria for RA[11] and had active disease. The joints that were selected for joint replacement did not exhibit active arthritis activity on clinical examination. Arthroscopy was not performed in patients who had a history of previous joint surgery. All patients gave informed consent, and the study protocol was approved by the medical ethics committees.

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Table 1 Demographic and clinical data of the 23 patients with rheumatoid arthritis (RA) who were studied for differences in cell infiltrate, and expression of matrix metalloproteinases (MMPs), angiogenic growth factors, and proinflammatory cytokines in ST selected at arthroscopy compared with joint surgery

Patient ID	Type	Age (years)	Sex (M/F)	Disease duration (months)	Disease modifying antirheumatic drugs	CRP (mg/l)	RF (+/−)	Erosions (+/−)
1	Arthroscopy	41	M	84	0	38	+	−
2	Arthroscopy	45	F	10	2	45	+	+
3	Arthroscopy	67	F	10	2	11	+	+
4	Arthroscopy	56	F	72	2	55	+	+
5	Arthroscopy	47	M	288	2	9	−	+
6	Arthroscopy	72	M	276	2	32	−	+
7	Arthroscopy	54	M	60	2	45	+	+
8	Arthroscopy	44	F	42	1	22	+	−
9	Arthroscopy	30	M	72	4	54	−	+
10	Arthroscopy	69	F	48	0	9	+	+
11	Arthroscopy	60	M	132	3	35	+	+
12	Arthroscopy	57	F	180	3	42	+	−
13	Arthroscopy	67	F	48	2	19	−	+
14	Surgery	31	M	72	4	54	−	+
15	Surgery	38	F	188	1	35	−	+
16	Surgery	64	M	132	1	126	+	+
17	Surgery	74	F	76	1	7	−	+
18	Surgery	60	F	300	6	37	+	+
19	Surgery	75	F	60	1	39	+	+
20	Surgery	71	F	240	1	16	+	+
21	Surgery	72	M	132	1	66	+	+
22	Surgery	67	F	168	2	29	−	+
23	Surgery	82	F	264	1	108	+	+

Synovial tissue

ST samples were obtained from different sites in the joint of patients with RA with destructive disease during joint replacement. In the second group of patients with RA with active arthritis of the knee joint, synovial biopsy specimens were selected from various regions by arthroscopy (2.7 mm arthroscope, Storz, Tuttlingen, Germany) under local anaesthesia.[12] The samples were snap frozen en bloc in Tissue-Tek OCT (Miles Diagnostics, Elkhart, IN). The frozen blocks were stored in liquid nitrogen. Cryostat sections (5 µm) were mounted on glass slides (Star Frost adhesive slides, Knittelgläser, Braunschweig, Germany). The glass slides were sealed and stored at −70°C until immunohistological analysis.

Immunohistochemistry

Serial sections were stained with the following mouse monoclonal antibodies (mAbs): anti-CD3 (Becton Dickinson, San Jose, CA), anti-CD4 (Leu-3a, Becton Dickinson), anti-CD8 (DK25, DAKO, Glostrup, Denmark), anti-CD38 (Leu-17, Becton Dickinson), anti-CD68 (EBM11, DAKO), anti-CD55 (mAb67, Serotec, Oxford UK), anti-MMP-1, which recognises both latent and active MMP-1 (36665.111, R&D Systems Europe Ltd, Abingdon, UK), anti-MMP-3, which recognises both latent and active MMP-3 (10D6, R&D), anti-MMP-13, which recognises both latent and active MMP-13 (181–15A12, Oncogene Research products, Cambridge, MA), anti-tissue inhibitor of metalloproteinase (TIMP)-1 (7–6C1, Oncogene Research products), and anti-vascular endothelial growth factor (anti-VEGF; C-1, Santa Cruz Biotechnology, Santa Cruz, CA). Staining was also done with the following rabbit polyclonal antibodies: anti-tumour necrosis factor α (anti-TNFα; IP-300, Genzyme, Cambridge, MA), anti-interleukin (IL)1β (LP-712, Genzyme), and anti-IL6 (LP-716, Genzyme). For control sections, the primary antibodies were omitted or irrelevant isotype matched mouse antibodies or rabbit immunoglobulins were applied.

Endogenous peroxidase activity was inhibited by 0.1% sodium azide and 0.3% hydrogen peroxide in phosphate buffered saline. Staining for cell markers and cytokines was performed, as described previously.[13] After a primary incubation step with mAbs, bound antibody was detected by a three step immunoperoxidase method. Alkaline phosphatase conjugated swine antirabbit antibodies (DAKO), naphthol-AS-MX-phosphate (N-AS-MX-P), fast red violet LB (FRVLB), and levamisole (Sigma) were used for the detection of the rabbit polyclonal antibodies. Staining for MMP-1, MMP-3, MMP-13, and TIMP-1 was performed using biotinylated tyramide for amplification, as described previously.[14] The primary antibodies were incubated for 60 minutes. Horseradish peroxidase (HRP) conjugated goat antimouse was added for 30 minutes, followed by subsequent incubation with biotinylated tyramide for 30 minutes and HRP conjugated streptavidin for 30 minutes. HRP activity was detected using hydrogen peroxide as substrate and 3-amino-9-ethylcarbazole as dye. Slides were counterstained with Mayer's haemalum solution (Merck, Darmstadt, Germany) and, after washing with distilled water, mounted in Kaiser's glycerol gelatin (Merck). Affinity purified and HRP conjugated goat antimouse antibody was obtained from DAKO, affinity purified HRP conjugated swine antigoat immunoglobulin from Tago (Burlingame, CA), biotinylated tyramide and HRP conjugated streptavidin were obtained from Nen Life Science Products (Boston, MA), and 3-amino-9-ethylcarbazole from Sigma (St Louis, MO).

Microscopic analysis

After immunohistochemical staining, coded sections stained for CD3, CD4, CD8, CD38, CD55, CD68, MMP-1, MMP-3, MMP-13, TIMP-1, VEGF, TNFα, IL1β, and IL6 were analysed in a random order by computer assisted image analysis. For all markers, 20 high power fields were analysed. The high power field images were analysed with the Qwin analysis system (Leica, Cambridge, England), as described previously in detail.[15 16]

Statistical analysis

The Mann-Whitney two sample test was used to compare the scores for the various markers from ST selected at arthroscopy with ST obtained at surgery. Because of the exploratory nature of the study, we did not correct for multiple comparisons.

Table 2 Mean integrated optical density (IOD) for the expression of CD3+, CD4+, and CD8+ T cells, CD38+ plasma cells, CD68+ macrophages, CD55+ FLS, TNFα, ILβ, IL6, VEGF, MMP-1, MMP-3, MMP-13, and TIMP-1 in ST obtained at arthroscopy compared with joint surgery from patients with rheumatoid arthritis (RA), and measured by computer assisted digital image analysis†

	Arthroscopy	Surgery
CD3	13804 (2851) [n=12]	6477 (2201) [n=10]
CD4	3172 (792) [n=13]	6627 (4141) [n=10]
CD8	3157 (908) [n=13]	4121 (2083) [n=10]
CD38	16154 (6297) [n=13]	14547 (6208) [n=9]
CD68 lining	8436 (1396) [n=13]	3797 (1669) [n=10]*
CD68 sublining	88847 (9443) [n=13]	44696 (10638) [n=10]*
CD55	10242 (2708) [n=13]	7146 (1457) [n=9]
TNFα	45868 (5224) [n=12]	40196 (5589) [n=10]
IL1β	3424 (859) [n=12]	7011 (1850) [n=10]
IL6	3023 (865) [n=9]	2277 (1207) [n=5]
VEGF	3705 (1461) [n=8]	2134 (914) [n=6]
MMP-1	618 (414) [n=13]	190 (97) [n=10]
MMP-3	18387 (6059) [n=13]	7559 (3474) [n=10]
MMP-13	3826 (2443) [n=9]	1465 (597) [n=6]
TIMP-1	5621 (2591) [n=8]	2521 (1272) [n=6]

*p<0.05.
†The data represent mean (SEM).

RESULTS

Clinical and demographic features

Table 1 presents the clinical and demographic data of the patients with RA included in the study. The study group with active RA undergoing arthroscopy comprised seven women and six men, with a mean (SD) age of 55 (13) years (range 30–72) and mean (SD) duration of disease of 102 (92) months (range 10–288). The mean (SD) serum level of C reactive protein was 32 (16) mg/l (range 9–55). Erosions were present in 10 of the 13 patients and nine patients were seropositive for IgM rheumatoid factor. Eleven of the 13 patients were treated with disease modifying antirheumatic drugs (DMARDs). The study group with end stage, destructive RA comprised seven women and three men, with a mean age of 63 (16) years (range 31–82 years) and mean duration of disease of 163 (84) months (range 60–300). The mean serum level of C reactive protein was 52 (39) mg/l (range 7–126). Erosions were present in all 10 patients and six patients were seropositive for IgM rheumatoid factor. All patients were treated with DMARDs.

In both groups, all patients used non-steroidal anti-inflammatory drugs; none used prednisolone.

Immunohistochemistry

The ST was characterised by intimal lining layer hyperplasia and accumulation of macrophages, T cells, plasma cells, and other cell types in the synovial sublining in both groups. Expression of cytokines and MMPs was on average abundant, especially for TNFα and MMP-3. Digital image analysis showed more pronounced macrophage infiltration in arthroscopic biopsy specimens than in ST obtained at surgery (mean (SEM) integrated optical density for CD68 in the intimal lining layer: 8436 (1396) in arthroscopic samples v 3797 (1669) in surgical samples (p<0.05); CD68 in the synovial sublining: 88 847 (9443) in arthroscopic samples v 44 696 (10 638) in surgical samples (p<0.01)) (table 2). In addition, there tended to be more T cells in arthroscopic biopsy specimens, but this difference did not reach significance (p=0.09). All other phenotypic markers showed similar expression patterns. The expression of TNFα, IL6, MMP-1, MMP-3, MMP-13, TIMP-1, and VEGF tended to be higher in biopsy specimens selected at arthroscopy than in surgically obtained ST (p>0.1). Of interest, IL1β was on average more abundant in destructive RA tissue than in arthroscopic biopsy specimens (p>0.1). These differences were not significant, presumably because of the relatively small number of patients in this study. There was no correlation between any of the scores and disease duration (data not shown).

DISCUSSION

The results presented in this study demonstrate differences in the immunohistological characteristics of arthroscopic synovial biopsy specimens compared with ST obtained at surgery. Statistically significant higher scores for infiltration by CD68+ macrophages in both the intimal lining layer and the synovial sublining were seen in arthroscopic samples. There also tended to be more CD3+ T cells in arthroscopic specimens than in surgical specimens. In addition, there was a tendency towards higher expression of TNFα, IL6, MMP-1, MMP-3, MMP-13, TIMP-1, and VEGF in ST selected at arthroscopy. In contrast, IL1β expression tended to be increased in end stage, destructive disease.

A theoretical limitation of this exploratory study is the risk of erroneously reporting statistically significant differences as a result of multiple comparisons. However, it seems unlikely that the observed differences between the two biopsy groups can be explained by chance only, because all immunohistological parameters related to inflammation were increased in the arthroscopy group. Furthermore, macrophage infiltration was increased in arthroscopic samples in both the intimal lining layer and the synovial sublining. These differences were highly significant. The only exception was IL1, which is consistent with previous reports,[17] suggesting a prominent role of IL1 in joint destruction.

In both biopsy groups we observed higher TNFα expression than IL1β expression. These results differ from previous reports.[18 19] The difference might be explained by the fact that here we used a method, which detects both cytokine producing cells and cytokine bound to cell receptors and extracellular matrix. Previous work showed a strong correlation between total TNFα protein detected with this method and disease activity.[6] Other studies, demonstrating relatively small amounts of TNFα in RA ST, have used a method showing cytokine producing cells only.[18] It is at present unknown which method represents disease mechanisms best, and both methods are used and have been validated.

Many previous studies have focused on ST, which was obtained from end stage, destructive RA during joint surgery.[3–5] Now that blind synovial biopsy and needle arthroscopy techniques are widely available, most of the current studies use ST from patients with active synovial

inflammation.[1][6][14] This study clearly shows that the features of ST are dependent on patient selection. This observation could be explained by several factors. Firstly, arthroscopic samples are usually obtained from clinically inflamed joints, whereas joint replacement is indicated in patients with cartilage and bone damage. In these patients synovial inflammation is not necessarily an important characteristic. The increase in macrophage infiltration in arthroscopic biopsy specimens is not unexpected in light of the significant correlation between scores for local arthritis activity and the number of macrophages in rheumatoid ST.[6] Secondly, the inflammatory response in end stage RA may be in part different from the initial disease process, because of the non-specific effect of secondary release of bone and articular cartilage on synovial inflammation.[20] Thirdly, it is conceivable that patients with destructive RA represent a specific pathogenetic subset. In these patients mechanisms other than those associated with clinical signs of inflammation may be more important, for example changes in the phenotype and behaviour of FLS[10][21] and increased IL1 expression.[17][22] IL1 inhibits both chondrocyte proteoglycan and collagen synthesis and stimulates the chondrocytes to release cartilage destructive proteases.

When patients are matched for disease activity, the features of arthroscopic biopsies are similar in early RA and longstanding disease.[6][23] Thus, so-called early RA already represents a chronic phase of the disease. This study clearly shows that when patients are not selected on the basis of arthritis activity, the results may be different. Previous studies on surgical specimens have for example described specific characteristics of the cellular composition at the cartilage-pannus junction.[24] However, recent systematic comparisons did not show any differences in cellularity and expression of MMPs in ST obtained from the cartilage-pannus junction compared with adjacent pannus.[14][25] The data presented here support the view that the differences in cellularity, expression of cytokines, and proteolytic enzyme between cartilage-pannus junction and adjacent pannus reflect the stage of the disease and disease activity rather than the specific site.

Taken together, this study shows the importance of patient selection for the interpretation of studies on rheumatoid ST. It can be expected that immunohistological methods, in situ hybridisation, the polymerase chain reaction, and cDNA microarray technology will increasingly be used for ST analysis. For these studies it will be essential to use biopsy samples from patients in an active stage of the disease to provide insight into the mechanisms underlying synovial inflammation.

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Authors' affiliations
T J M Smeets, M C Kraan, P P Tak, Division of Clinical Immunology and Rheumatology, Academic Medical Centre/University of Amsterdam, Amsterdam, The Netherlands
E C Barg, F C Breedveld, Department of Rheumatology, Leiden University Medical Centre, Leiden, The Netherlands
M D Smith, Rheumatology Research Unit, Repatriation General Hospital, Daw Park, SA, Australia

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