EXTENDED REPORTS

Analysis of the cell infiltrate and expression of matrix metalloproteinases and granzyme B in paired synovial biopsy specimens from the cartilage-pannus junction in patients with RA

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Abstract

Objectives—Examination of synovial tissue (ST) obtained at surgery because of end stage destructive rheumatoid arthritis (RA) showed that macrophages and fibroblasts are the major cell types at the cartilage-pannus junction (CPJ). This study aimed at defining the cell infiltrate and mediators of joint destruction in ST selected at arthroscopy from the CPJ in patients with RA who did not require joint surgery.

Methods—Paired synovial biopsy specimens were obtained at arthroscopy from ST adjacent to the CPJ and the suprapatellar pouch from the knee joints of 17 patients with RA. Immunohistological analysis was performed using monoclonal antibodies to detect T cells, B cells, plasma cells, macrophages, fibroblast-like synoviocytes, mast cells, and granzyme B+ cytotoxic cells as well as the expression of metalloproteinase (MMP)-1, MMP-3, and MMP-13. The sections were evaluated by computer assisted image analysis and semiquantitative analysis.

Results—The cell infiltrate comprised mainly T cells, macrophages, and plasma cells. The ST was also infiltrated by the other cell types, but at lower numbers. Expression of MMPs was abundant, especially MMP-3. The features of ST at the CPJ were generally similar to those at the suprapatellar pouch.

Conclusions—The synovium at the CPJ in patients with RA who did not require joint surgery exhibits, in general, the same type of cell infiltrate and expression of MMPs and granzymes as ST from the suprapatellar pouch. The pathological changes that have been described at the CPJ in patients with RA with end stage, destructive disease may well reflect the transition to a process in which macrophages, fibroblast-like synoviocytes, and other cell types become increasingly important.

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Rheumatoid arthritis (RA) is characterised by synovial membrane inflammation, leading to invasion of synovial tissue (ST) into the adjacent cartilage matrix, with proteolytic degradation of articular cartilage and bone as a consequence. Previous histopathological studies on ST, obtained during therapeutic arthrotomy procedures on knee joints of patients with RA, showed that the majority of cell types at the cartilage-pannus junction (CPJ) consist of macrophages and fibroblasts.[1–6] Mast cells[7] and polymorphonuclear leucocytes[8] can also be found, but T cells are rarely found at the CPJ. Immunoreactive cells located at the CPJ from arthrotomy samples are often locally concentrated in different microfoci of the same junction.[1] In addition, the expression of the proinflammatory cytokines tumour necrosis factor and interleukin 1β has been described at the CPJ.[9–10] These cytokines can induce the synthesis of matrix metalloproteinases (MMPs) in fibroblast-like synoviocytes (FLS) and articular cartilage.[11–13] They are believed to induce degradation of cartilage and bone, and inhibit proteoglycan synthesis. It has previously been shown that cells expressing serine proteinase granzyme B are found both in ST at the CPJ[14] and in ST obtained from the suprapatellar pouch.[15] The levels of soluble granzyme B in synovial fluid[16] and the number of granzyme B positive cells in ST[15] are specifically increased in RA and may contribute to joint destruction by degrading proteoglycans.

This study aimed at defining the cell infiltrate and mediators of joint destruction in arthroscopically obtained ST from tissue adjacent to the CPJ, compared with paired ST obtained from the suprapatellar pouch.

Patients and methods

PATIENTS

Seventeen patients with active RA and arthritis of a knee joint were investigated. Nine patients from the Repatriation General Hospital in Daw Park, South Australia, and eight patients from the Leiden University Medical Centre were included. All patients fulfilled the American College of Rheumatology criteria for RA[17] and had active arthritis, defined as follows: six or more tender joints, six or more swollen joints,

Table 1 Demographic and clinical data of the eight patients with rheumatoid arthritis from Leiden and the nine patients from Daw Park, who were studied for variations in cell infiltrate, and expression of matrix metalloproteinases and granzyme B of synovial tissue from cartilage-pannus junction and suprapatellar pouch

Patient	Age (years)	Sex (M/F)	Disease duration (months)	DMARDs*	CRP* (mg/l)	RF* (+/−)	Erosions (+/−)
1	54	M	30	Plaquenil	14	−	+
2	55	M	276	—	37	−	−
3	48	F	84	MTX*	4	−	+
4	65	M	288	MTX	27	+	+
5	58	F	276	MTX	17	−	+
6	80	F	336	Prednisone	45	+	+
7	80	F	336	Prednisone	45	+	+
8	43	F	120	MTX, prednisone, cyclosporin	29	+	+
9	77	M	17	Gold	1	−	−
10	76	F	120	Cyclosporin	46	+	+
11	72	F	13	—	35	+	+
12	60	F	24	Plaquenil	80	−	−
13	77	M	180	Gold	16	+	+
14	70	M	1	Gold, MTX	116	+	−
15	75	F	3	Gold	47	+	−
16	76	F	1	Gold	32	−	−
17	59	F	216	MTX	108	+	+

*DMARDs = disease modifying antirheumatic drugs; CRP = C reactive protein; RF = rheumatoid factor, MTX = methotrexate.

and at least one of the following two criteria: duration of morning stiffness ⩾45 minutes, or an erythrocyte sedimentation rate ⩾28 mm/1st h. Laboratory assessment included the measurement of serum levels of C reactive protein and rheumatoid factors. All patients gave informed consent, and the study protocol was approved by the medical ethics committees.

SYNOVIAL TISSUE

A small-bore arthroscopy (2.7 mm arthroscope, Storz, Tuttlingen, Germany) was performed under local anaesthesia. Biopsy specimens of ST were obtained from each patient from the CPJ and the suprapatellar pouch. The samples were separately snap-frozen in Tissue-Tek OCT (Miles Diagnostics, Elkhart, IN, USA). 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 needed for immunohistological analysis.

IMMUNOHISTOCHEMISTRY

Serial sections were stained with the following mouse monoclonal antibodies (mAb): anti-CD3 (Becton Dickinson, San Jose, CA), anti-CD4 (Leu-3a, Becton Dickinson), anti-CD8 (DK25, DAKO, Glostrup, Denmark), anti-CD22 (CLB-B-Ly/1, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), anti-CD38 (Leu-17, Becton Dickinson), anti-CD68 (EBM11, DAKO), mAb 67, which recognises CD55, anti-mast cell tryptase (AA1, DAKO), 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), and anti-granzyme B7 (GrB-7, Sanbio, Uden, The Netherlands).

Endogenous peroxidase activity was inhibited using 0.1% sodium azide and 0.3% hydrogen peroxide in phosphate buffered saline.

Staining for cell markers was performed, as described previously.[18] After a primary incubation step with mAbs, bound antibody was detected by a three step immunoperoxidase method. Staining for MMP-1, MMP-3, MMP-13, and granzyme B7 was performed using biotinylated tyramine for amplification. 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 tyramine for 30 minutes and HRP conjugated streptavidin for 30 minutes. HRP activity was detected using hydrogen peroxide as substrate and aminoethylcarbazole 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 anti-goat immunoglobulin from Tago (Burlingame, CA), biotinylated tyramine and HRP conjugated streptavidin were obtained from Nen Life Science Products (Boston, MA), and aminoethylcarbazole from Sigma (St Louis, MO).

MICROSCOPIC ANALYSIS

After immunohistochemical staining, coded sections for CD3, CD4, CD8, CD22, CD38, CD68, MMP-1, and granzyme B7 were analysed in a random order by computer assisted image analysis. For CD3, CD4, CD8, CD38, CD68, and MMP-1, 20 high power fields (HPFs), and for CD22 and granzyme B7, six HPFs were analysed. The HPF images were analysed using the Qwin analysis system (Leica, Cambridge, England).[19] Coded sections for CD3, CD4, CD8, CD22, CD38, CD68, CD55, mast cells, MMP-1, MMP-3, and MMP-13 were also scored semiquantitatively on a five point scale by two independent observers in a random order, as described previously.[18–20] The number of granzyme B7 positive cells were each recorded in every HPF (×312.5 magnification). A five point scale was

Table 2   Mean semiquantitative scores for the expression of CD3+, CD4+, and CD8+ T cells, CD22+ B cells, CD38+ plasma cells, CD68+ macrophages, CD55+ fibroblast-like synoviocytes, and the expression of mast cells, matrix metalloproteinases MMP-1, MMP-3, MMP-13, and granzyme B7 in synovial tissue derived from cartilage-pannus junction (CPJ) and suprapatellar pouch (non-CPJ) from patients with rheumatoid arthritis. The data are shown as means (SD)

	n	CPJ	Non-CPJ
CD3 (aggregates)	17	1.5 (0.7)	1.5 (0.8)
CD3 (diffuse)	17	2.9 (1.0)	2.7 (1.0)
CD4	17	2.5 (0.9)	2.2 (1.0)
CD8	16	2.3 (0.8)	2.3 (1.1)
CD22	17	2.3 (0.9)	1.9 (0.7)
CD38	17	2.5 (1.3)	2.3 (1.3)
CD68 (lining)	16	2.1 (0.7)	1.9 (1.1)
CD68 (sublining)	16	3.1 (0.9)	2.8 (0.8)
CD55	17	1.7 (0.7)	1.4 (0.5)
Mast cells	16	1.9 (0.8)	1.9 (0.9)
MMP-1	15	1.7 (1.0)	1.8 (1.3)
MMP-3	15	2.6 (1.4)	2.3 (1.4)
MMP-13	15	2.5 (0.5)	2.5 (1.0)
Granzyme B7	15	1.3 (1.0)	1.5 (1.3)

used: 0=⩽0.5, 1=0.6–1.5, 2=1.6–5.0, 3=5.1–9.0, and 4=⩾9.1 mean granzyme B+ cells per HPF.

STATISTICAL ANALYSIS
The Wilcoxon signed ranks test for matched pairs was used to compare synovial tissue derived from the CPJ and the suprapatellar pouch. In addition, the Spearman rank correlation between immunohistological scores and disease duration in the CPJ group was calculated (data not shown).

Results

CLINICAL AND DEMOGRAPHIC FEATURES
Table 1 presents clinical and demographic data of the patients with RA included in the study. The study group comprised 11 women and six men, with a mean (SD) age of 66 (12) years (range 43–80) and a mean disease duration of 120 (125) months (1–336). The mean (SD) serum level of C reactive protein was 43 (33) mg/l (1–116). Erosions were present in 11/17 patients, and 10 patients were seropositive for
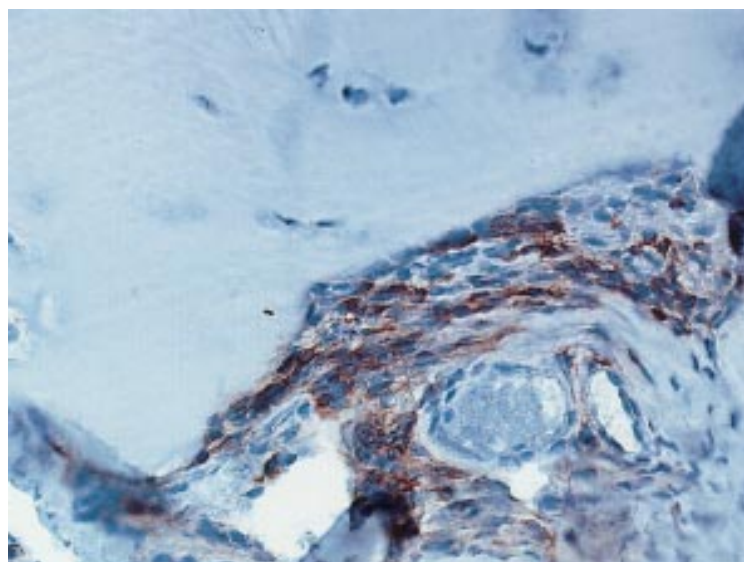
Figure 1   CD3 positive T cells localised at the cartilage-pannus junction in arthroscopically derived rheumatoid synovial tissue. Single stain peroxidase technique; Mayer's haemalum counterstained.

Table 3   Mean counts of CD3+, CD4+, and CD8+ T cells, CD22+ B cells, CD38+ plasma cells, CD68+ macrophages, and the integrated optical density (IOD) of matrix metalloproteinase-1 (MMP-1) and granzyme B7 in synovial tissue derived from cartilage-pannus junction (CPJ) and suprapatellar pouch (non-CPJ) from patients with rheumatoid arthritis, and measured by computer assisted digital image analysing. Results are shown as means (SEM)

	n	CPJ	Non-CPJ
CD3 (count)	17	1 228 (208)	1 278 (245)
CD4 (count)	17	441 (91)	437 (85)
CD8 (count)	16	144 (47)	260 (143)
CD22 (count)	17	202 (41)	127 (25)
CD38 (count)	17	1 411 (260)	1 173 (231)
CD68 sublining (count)	16	2 968 (209)	2 654 (249)
Granzyme B (IOD)	15	644 (136)	703 (131)
MMP-1 (IOD)	15	71 749 (11 356)	63 850 (9 280)

IgM rheumatoid factor. All patients were treated with non-steroidal anti-inflammatory drugs and 15/17 patients were treated with disease modifying antirheumatic drugs.

IMMUNOHISTOCHEMISTRY
The semiquantitative scores for infiltration by inflammatory cells and the scores for expression of mast cells, MMP-1, MMP-3, MMP-13, and granzyme B in ST of patients with RA were, on average, similar when the ST biopsy specimens derived from the CPJ were compared with those derived from the suprapatellar pouch (p>0.1) (table 2). Of interest, T cells were also seen at the CPJ (fig 1). There was no correlation between the semiquantitative scores and disease duration, independent of the site of biopsy (data not shown). In addition, digital image analysis showed that the mean counts for CD3, CD4, CD8, CD22, CD38, and CD68, as well as the integrated optical density for granzyme B and MMP-1 in ST biopsy specimens derived from the CPJ, were similar to those derived from the suprapatellar pouch, though there tended to be more CD22+ B cells and fewer CD8+ T cells at the CPJ (table 3).

Discussion
The results presented in this study show that the features of synovial biopsy specimens of patients with active RA, who did not (yet) require joint surgery, are similar at the CPJ and in the suprapatellar pouch. Infiltration by macrophages, plasma cells, T cells, B cells, and mast cells was clearly present at both sites. In addition, there was marked expression of latent or active MMP-1, MMP-3, MMP-13, and granzyme B. The observations using semi-quantitative analysis were confirmed by digital image analysis, which may be more sensitive to detect small differences than semiquantitative analysis.

The cellular composition along the CPJ shows many variations.[21] The CPJ has been described as relatively acellular in some patients with destructive RA.[1–2] The cells occupying the CPJ may show a different cellular density and morphology than those of the adjacent synovial tissue.[21] The relatively high expression of collagenase along the CPJ, compared with the adjacent pannus tissue[22] as well as the presence of myeloid related proteins, particularly at

the CPJ,[23] suggest differences in physiological conditions between the CPJ and nearby pannus tissue. Furthermore, a recent study indicated more pronounced macrophage infiltration in biopsy specimens obtained by needle arthroscopy from tissue adjacent to the CPJ than in samples obtained by blind needle biopsy from the suprapatellar pouch.[24] These observations suggest that synovial biopsy specimens taken from different sites of the joint may reflect differences in cellularity as well as differences in expression of cytokines and proteolytic enzymes.

In most studies examining the CPJ, synovial tissue was obtained from end stage, destructive RA during joint surgery.[9–26] In these patients inflammation is not necessarily a prominent feature. Of importance, there is a significant relation between the features of synovial inflammation and local disease activity.[27–28] Therefore, the selection of patients may strongly influence the characteristics of the synovium.

Intimal lining layer hyperplasia seems to be less prominent in arthroscopic samples[27] than in tissue obtained at surgery, though this has not been studied systematically. In line with this hypothesis p53 expression is especially prominent in the intimal lining layer of patients with RA with end stage, destructive disease,[29] whereas p53 expression is more pronounced in the synovial sublining of samples obtained by arthroscopy from patients with active RA.[30] A recent study showed p53 mutations in FLS from the synovium of patients with RA with longstanding, destructive RA, but not in FLS from German patients with RA.[31] It has been suggested that this difference may be explained by differences in disease duration and disease severity, suggesting that p53 mutations can be seen in longstanding, destructive disease but not in earlier phases of RA.[32] The p53 mutations might explain the "transformed" phenotype of FLS[33] and their selective advantage as a result of increased proliferation and impaired apoptosis.[34] FLS and macrophages produce a variety of soluble mediators, which are thought to play a part in joint destruction. Consequently, increased numbers of these cells at the CPJ of patients with RA who require joint surgery would not be unexpected. Previous studies showed that T cells were rarely found in CPJ from end stage RA synovial tissue. The demonstration of T cells at the CPJ in this study may reflect the importance of T cells in perpetuating the disease process in the early and middle phases of the disease, whereas they might become less important in joint degradation at late disease stages.

In this study we compared the CPJ and suprapatellar pouch in paired biopsy samples from patients with active arthritis, but who did not require joint replacement. In both areas active inflammation could be shown. There were no significant differences between the two sites. These observations confirm and extend a recent study of intra-articular variability in the synovium of patients with RA.[35] The investigators found no systematic difference in the number of macrophages and T cells or in the cytokine expression at different sites of the knee joint.

However, we cannot completely exclude the possibility that there are a few cell layers adjacent to the cartilage, where the tissue is characterised by increased accumulation of macrophages, fibroblast, PMNs, and mast cells.

Taken together, this study shows that examination of RA ST from different sites in the knee joint discloses no differences in the composition of the cell infiltrate and the expression of MMPs and granzymes in the synovial tissue between the specific sites. The pathological changes that have been described at the CPJ in patients with RA with end stage destructive disease may well reflect the transition to a process in which macrophages, fibroblasts, and other cell types become increasingly important.

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