Activation of the STAT1 pathway in rheumatoid arthritis

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EXTENDED REPORT

Background: Expression of signal transducer and activator of transcription 1 (STAT1), the mediator of interferon (IFN) signalling, is raised in synovial tissue (ST) from patients with rheumatoid arthritis (RA). The objective of this study was to determine the extent to which this pathway is activated by phosphorylation in RA synovium. Additionally, we investigated the cellular basis of STAT1 activation in RA ST.

Methods: ST specimens from 12 patients with RA and 14 disease controls (patients with osteoarthritis and reactive arthritis) were analysed by immunohistochemistry, using antibodies to STAT1, tyrosine phosphorylated STAT1, and serine phosphorylated STAT1. Lysates of cultured fibroblast-like synoviocytes stimulated with IFNγ were analysed by western blotting. Phenotypic characterisation of cells expressing STAT1 in RA ST was performed by double immunolabelling for STAT1 and CD3, CD22, CD55, or CD68.

Results: Raised levels of total STAT1 protein and both its activated tyrosine and serine phosphorylated forms were seen in RA synovium as compared with controls. STAT1 was predominantly abundant in T and B lymphocytes in focal inflammatory infiltrates and in fibroblast-like synoviocytes in the interliminal layer. Raised levels of STAT1 were sustained in cultured RA compared with OA fibroblast-like synoviocytes, and STAT1 serine and tyrosine phosphorylation is rapidly induced upon stimulation with IFNγ.

Conclusion: These results demonstrate activation of the STAT1 pathway in RA synovium by raised STAT1 protein expression and concomitantly increased tyrosine (701) and serine (727) phosphorylation. High expression of STAT1 is intrinsic to RA fibroblast-like synoviocytes in the interliminal layer, whereas activation of the pathway by phosphorylation is an active process.

A well-characterised feature of rheumatoid arthritis (RA) synovial tissue (ST) is a marked increase in cellularity. Factors that contribute to ST hyperplasia are recruitment and retention of inflammatory cells, enhanced cell proliferation, and impaired apoptosis. Cytokines, derived from activated synovial cells, have a pivotal role in the regulation of these processes.

Critical in mediating virtually all cytokine-driven signalling are the members of the STAT (signal transducer and activator of transcription) family of transcription factors, which are involved in regulating gene expression and cellular activation, proliferation, and differentiation.

Recently, we and others demonstrated raised expression of STAT1 mRNA and protein in the ST of patients with RA as compared with disease controls, indicating that this pathway might be involved in RA pathogenesis. STAT1 is essential for interferon (IFN) signalling. IFNs are typically growth inhibitory and promote immune recognition of target cells, and absence of IFN/STAT1 signalling is associated with RA pathogenesis. STAT1 protein expression and phosphorylation are decreased in RA synovium compared with ST from disease controls, including patients with osteoarthritis (OA) and reactive arthritis (ReA). In addition, we characterised the expression and activation of STAT1 in the various cell types in RA ST.

MATERIALS AND METHODS

Patients
ST was obtained from the actively inflamed knee joints of 26 patients, including 12 patients with RA (1987 American College of Rheumatology (ACR) criteria), 7 patients with inflammatory OA (1987 criteria), and 7 patients with ReA.

All material was obtained in the early 1990s; at that time ST could be obtained from all patients before disease-modifying antirheumatic drug (DMARD) treatment was initiated.

Knee joint arthroscopy and synovial biopsies
In all patients an arthroscopy was performed under local anaesthesia using a small bore, 2.7 mm arthroscope (Storz)

Abbreviations: ACR, American College of Rheumatology; AP, alkaline phosphatase; BSA, bovine serum albumin; DMARD, disease-modifying antirheumatic drug; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HPF, high power field; HRP, horseradish peroxidase; IFN, interferon; IFM, immunofluorescence microscopy; IOD, integrated optical density; OA, osteoarthritis; RA, rheumatoid arthritis; ReA, reactive arthritis; ST, synovial tissue; STAT, signal transducer and activator of transcription
and using an infrapatellar skin portal for macroscopic examination of the synovium and a second suprapatellar portal for the biopsy procedure.15 Synovial biopsy samples were obtained from multiple regions (>6) using a 2.5 mm grasping forceps (Storz). Tissue samples were embedded en bloc in Tissue Tek OCT compound (Miles Diagnostics, Elkhart, IN) and snap frozen in liquid nitrogen. The frozen tissue were stored in liquid nitrogen until sectioned for staining. Sections (5 μm) were cut in a cryostat and mounted on glass slides (Star Frost adhesive slides, Knittelgläser, Germany); the slides were air dried at room temperature, carefully packed, and sealed and stored at −80°C until immunohistochemical staining was performed.

Antibodies

Serial sections were stained using the following monoclonal antibodies and diluted as indicated for immunohistochemistry (IHC) and immunofluorescence microscopy (IFM): anti-STAT1 (tyrosine (Y701)), 1:160 (BD Transduction Laboratories); anti-phospho-STAT1-tyrosine (Y701), 1:40 (Zymed Laboratories Inc, San Francisco, CA); anti-CD3, CD22, CD55, CD68, followed by rabbit-anti-FITC (DAKO) and swine-isothiocyanate (FITC) conjugated anti-CD3, -CD22, -CD55, or -CD68, followed by rabbit-anti-FITC (DAKO) and swine-isothiocyanate (FITC) conjugated anti-CD3, -CD22, -CD55, or -CD68. For double IHC, sections were incubated with anti-STAT1 followed by anti-goat-HRP for 30 minutes, followed by swine-antimouse antibody, sections were blocked with 10% normal goat serum (Sanquin) for 15 minutes. Subsequently, antimouse was added for 30 minutes, followed by swine-antigoat-HRP for 30 minutes. To prevent binding to excess antimouse antibody, sections were blocked with 10% normal mouse serum (Sanquin) for 15 minutes. Subsequently, sections were incubated for 60 minutes with fluorescein isothiocyanate (FITC) conjugated anti-CD3, -CD22, -CD55, or -CD68, followed by rabbit-anti-FITC (DAKO) and swine-antirabbit-AP, each for 30 minutes. HRP activity was detected using hydrogen peroxidase as substrate and aminoethylcarbazole as dye (Sigma, St Louis, MO). AP activity was detected using a three step alkaline phosphatase (AP) method for CD3, CD22, CD55, and CD68. An immunoperoxidase method was used for STAT1 and phospho-STATs. For these antibodies, except for STAT1, staining was performed using biotinylated tyramine (Perkin Elmer Life Sciences, Boston, MA) for amplification.

For double IHC, sections were incubated with anti-STAT1 overnight. Horseradish peroxidase (HRP) conjugated goat-antimouse was added for 30 minutes, followed by swine-antigoat-AP conjugated horse radish peroxidase (HRP) for 30 minutes. To prevent binding to excess antimouse antibody, sections were blocked with 10% normal mouse serum (Sanquin) for 15 minutes. Subsequently, sections were incubated for 60 minutes with fluorescein isothiocyanate (FITC) conjugated anti-CD3, -CD22, -CD55, or -CD68, followed by rabbit-anti-FITC (DAKO) and swine-antirabbit-AP, each for 30 minutes. HRP activity was detected using hydrogen peroxidase as substrate and aminoethylcarbazole as dye (Sigma, St Louis, MO). AP activity was detected using a three step alkaline phosphatase (AP) method for CD3, CD22, CD55, and CD68. An immunoperoxidase method was used for STAT1 and phospho-STATs. For these antibodies, except for STAT1, staining was performed using biotinylated tyramine (Perkin Elmer Life Sciences, Boston, MA) for amplification.

Digital image analysis

Sections analysed by immunohistochemistry were coded and assessed by digital image analysis in random order as described previously.16 Briefly, three separate representative regions, including the intimal lining layer and synovial sublining, were chosen for the evaluation of each section. Six consecutive high power fields (HPFs) from each region were captured and digitised, resulting in a total of 18 HPFs (surface area ~2.1 mm²). Subsequently, sections were

Table 1  Demographic and clinical data of the 12 patients with RA and the control patients, including seven patients with OA and seven patients with ReA, who were studied for expression of STAT1 and its tyrosine and serine phosphorylated forms. All material was collected in the early 1990s; at that time synovial tissue could be obtained from all patients before DMARD treatment was initiated.

<table>
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examined using a specialised algorithm written in the program language QWIPS operating a Qwin based (Qwin Pro V2.4; Leica, Cambridge, UK) computer assisted, colour video image analysis system. 20 To quantify staining for STAT1, STAT1-P-Tyr, and STAT1-P-Ser in the analysed regions, integrated optical density (IOD) was calculated as the product of staining area and intensity 17 and presented as IOD/mm² tissue. The total cell count of the measured regions was determined by quantification of nuclear counterstaining, which allowed for normalisation for cellularity in all regions.

**Fluorescence microscopy**

Double immunofluorescence was performed on sections of four patients with RA. The staining procedure used is a modification of the method described previously. 20 Firstly, sections were incubated with anti-STAT1 monoclonal antibody, followed by an antimouse isothiocyanate (TRITC) conjugated goat-antimouse IgG1 antibody (Nordic, Tilburg, The Netherlands). After use of 10% normal mouse serum (Sanquin) as blocking serum, incubation with FITC labelled anti-CD3, -CD22, -CD55, or -CD68 was performed on serial sections. Sections were embedded in mounting medium (Vectorshield, Vector Labs Inc) and analysed by two independent assessors (PVK and TJS). During the analysis, all cells that were double positive for STAT1 and the respective CD marker were counted and given as a percentage of the absolute number of either CD3+, CD22+, CD55+, or CD68+ cells in the biopsy specimens.

**Cell culture and stimulation**

Fibroblast-like synoviocytes were obtained from synovial biopsy samples of patients with RA who fulfilled the criteria of the ACR 15 and patients with OA (1987 criteria 16). After enzymatic digestion, fibroblast-like synoviocytes were grown in culture flasks in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS), and cells were routinely split. Experiments were performed using third to sixth passage fibroblast-like synoviocytes. At this time, <2% contaminating lymphocytes, NK cells, or macrophages were present. The day before an experiment, cells were replated in medium containing 0.5% FCS. Stimulation was performed for 30 minutes in six-well dishes using recombinant human IFNβ (Sanquin) at a final concentration of 250 U/ml. Cell extracts were prepared in ice cold RIPA buffer (20 mM Tris; 150 mM NaCl; 1% sodium deoxycholic acid; 1% NP-40; 0.1% sodium dodecyl sulphate; 50 mM NaF; 250 μM Na3VO4; 10 mM Na2HPO4; 1 pill/30 ml of Complete (Roche) protein inhibitor) and kept on ice on a shaking platform for 30 minutes. After centrifugation at 4000 rpm for 10 minutes, supernatants were transferred to a fresh tube and frozen at −20°C until further use.

**Western blot analysis**

Cell lysates of fibroblast-like synoviocytes were corrected for total protein content (35 μg total protein per lane) and fractionated on 7.5% sodium dodecyl sulphate-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in Tris buffered saline-Tween/3% bovine serum albumin (BSA) for 1 hour and then incubated with the antibodies described above that were directed against STAT1, phospho-STAT1-tyrosine (Y701), or phospho-STAT1-serine(S727) at a dilution of 1:500 in Tris buffered saline-Tween/1% BSA. Enhanced chemiluminescence was used for detection.

**Statistical analysis**

The Mann-Whitney two sample test was used for comparison of RA ST with control ST.

**RESULTS**

**Activation of the STAT1 pathway in RA synovium**

To obtain insight into the extent of activation of the STAT1 pathway in RA synovium, immunohistochemical studies were performed on the ST of 12 patients with RA and 14 disease controls, including 7 patients with OA and 7 with ReA (table 1). Only patients who had not yet been treated with DMARDs were included in the study, and the RA patient group consisted of patients with both early and longstanding RA. Serial sections were stained for STAT1 and both its phosphorylated forms, phospho-STAT1(Y701) and phospho-STAT1(S727). STAT1 expression and phosphorylation was predominantly abundant in the intimal lining layer and in focal inflammatory infiltrates in the synovial sublining (fig 1A).

To make a quantitative comparison between the patients with RA and the control group, expression was quantified by digital image analysis. 20 Comparison of RA ST with ST of disease controls showed that STAT1 protein expression was raised in RA ST (expressed as mean (SD) IOD per mm² of tissue, 209 (214) v 24 (29) in disease controls, p = 0.0097, data not shown), which is consistent with our previously reported results. 5 Normalisation to the total number of cells present in the sections shows that the difference is still highly significant (p = 0.014), indicating that this effect could not merely be explained by differences in leucocyte infiltration. In addition, we found that STAT1 phosphorylation on tyrosine was significantly increased in RA ST as compared with controls (p = 0.017) as well as phosphorylation on serine (p = 0.029) (fig 1B). Calculation of the ratio of phosphorylated STAT1 to total STAT1 present for each patient showed no significant differences between patients with RA and disease controls (p = 0.70 and p = 0.52 for STAT1-P-Tyr/STAT1 and STAT1-P-Ser/STAT1, respectively), which suggests that activation by phosphorylation may increase concomitantly with STAT1 expression.

**STAT1 is differentially expressed in RA synovial tissue**

To evaluate specifically which cells in the different synovial compartments express STAT1, immunohistochemical double labelling was performed on the ST of four patients with RA using antibodies to detect T cells (anti-CD3), B cells (anti-CD22), fibroblast-like synoviocytes (anti-CD55), and macrophages (anti-CD68).

In the synovial sublining, STAT1 was mainly localised in both B and T lymphocytes (figs 2A and 2B). Focal inflammatory infiltrates of CD22+ B cells were found that exhibited strongly positive staining for STAT1 (fig 2A). High STAT1 expression was also seen in CD3+ T lymphocytic aggregates. Interestingly, in one patient both STAT1 positive T lymphocytic aggregates and T lymphocytic aggregates that were entirely negative for STAT1 were seen (fig 2B). CD68+ macrophages were abundant around lymphocyte clusters and in the intimal lining layer. Regardless of their position in the tissue, staining for STAT1 was relatively limited in these cells (fig 2D). In contrast, CD55+ fibroblast-like synoviocytes in the intimal lining layer stained strongly positive for STAT1 (fig 2C).

To make a quantitative assessment of the percentage of STAT1+ cells in these synovial cell populations, double immunofluorescence was performed (fig 3A) on the ST of four patients with RA who were not included in the previous analysis. The results confirmed the observation that STAT1 is expressed by B lymphocytes (fig 3B; mean (SD) STAT1+ cells of total CD22+ cells = 45 (5)%), T lymphocytes (54 (8)%), and the majority of fibroblast-like synoviocytes (87 (9)%). Only a limited number of macrophages (18 (15)%) were found to be STAT1+. Taken together, these results indicate
Figure 1  Increased STAT1 phosphorylation on tyrosine and serine in RA ST as compared with controls. Serial ST sections from 12 patients with RA and 14 controls were stained for STAT1-phospho-tyrosine and STAT1-phospho-serine using aminoethylcarbazole as dye (A) representative sections are shown. Counterstaining was performed using Mayer's haematoxylin. (Original magnification ×400.) Sections were analysed using computer assisted digital image analysis (B). Results are shown as means, errors bars indicate SD, on the y axis the mean IOD per mm² of tissue (see “Materials and methods”) is shown.

Figure 2  Immunohistochemical double labelling of the ST of four patients with RA (table 1; patients Nos 4, 5, 7, and 13; representative sections are shown) for STAT1 and CD22+ B cells (A), CD3+ T cells (B), CD55+ fibroblast-like synoviocytes (C), or CD68+ macrophages (D). STAT1 was detected using aminoethylcarbazole as dye (red) and CD molecules were detected using AP substrate (blue); no counterstaining was performed (see “Materials and methods”). (Original magnification ×400.)
that the abundance of STAT1 in the intimal lining layer is predominantly due to expression by fibroblast-like synoviocytes.

**STAT1 expression and activation in fibroblast-like synoviocytes**

To validate our in vivo observations, we assessed the expression of STAT1 in RA and OA fibroblast-like synoviocytes by immunoblot analysis of lysates of cultured cells. These studies disclosed higher expression of STAT1 in RA fibroblast-like synoviocytes than in OA synoviocytes (fig 4), which is consistent with our in vivo observations in whole ST. Interestingly, although expression of STAT1 was sustained in vitro, tyrosine phosphorylation was absent and only low basal levels of serine phosphorylation were found in cultured fibroblast-like synoviocytes from both patients with RA and OA. Stimulation with IFNγ rapidly induced both tyrosine and serine phosphorylation. These results show that whereas raised expression of STAT1 is a stable, intrinsic feature of RA fibroblast-like synoviocytes, whereas activation by phosphorylation is an active process.

**DISCUSSION**

The prime aim of the present study was to extend our understanding of the expression and activation of STAT1 in RA. Previously, we and others reported that STAT1 expression is raised in RA synovium as compared with controls, and STAT1 activation has been shown in rheumatoid synovial fluid cells. In the present work we provide evidence for activation of the STAT1 pathway in RA synovium and define the cell types that express STAT1. Raised expression of STAT1 appears to be intrinsic to RA fibroblast-like synoviocytes, whereas activation by phosphorylation is an active process.

Regulation of signalling through the Jak-STAT pathway is complex and occurs at several levels within the cell. Upregulation of STAT1 mRNA and protein expression in response to stimulation with IFNs has been described previously. Key post-translational modifications include tyrosine and serine phosphorylation, which are required for dimerisation and full transcriptional activity of STATs. Our results demonstrate that activation of STAT1 in the RA inflamed joint is not only reflected by increased expression of STAT1 but also by increased phosphorylation on both tyrosine (701) and serine (727) residues. The fact that
STAT1 activation is less pronounced in patients with OA and ReA may reflect the differential expression of IFNβ and IFNγ between diseases. These findings are in line with our microarray results, which demonstrated an increased expression of IFN-induced genes in RA STs that display up regulation of STAT1 mRNA. The observation that ratios of phosphorylated STAT1 to total STAT1 for each patient are comparable in patients with RA and controls suggests that up regulation of STAT1 protein expression and its activation by phosphorylation are concomitant events. Whether this is really true, however, can only be determined if extensive studies at the single cell level are performed.

In accordance with our microarray gene profiling results, we observed considerable variation in STAT1 expression and activation among patients with RA and controls. In the patient group studied patients with both early and longstanding RA were included, and no correlations between STAT1 activation and the duration of disease or other clinical variables (table 1) were found. This, however, may be due to the relatively small number of patients studied. We investigated the expression profile of STAT1 in the different synovial compartments in patients with RA by using two different double labelling techniques. In the synovial sublining we detected increased STAT1 protein expression in focal inflammatory infiltrates of both B and T lymphocytic origins. Interestingly, in one patient we detected a STAT1 positive perivascular T lymphocytic aggregate (fig 3A, second panel). Although the majority of lymphocytic infiltrates in RA were STAT1 positive, in some aggregates STAT1 expression was entirely absent. This can be seen in fig 2, where a STAT1 positive T lymphocyte aggregate and an adjacent STAT1 negative aggregate are shown (fig 2B). These findings suggest that STAT1 is differentially expressed in synovial lymphocytes, which may reflect the state of activation and cellular differentiation of these cells.

STAT1 expression was also abundant in the intimal lining layer. The vast majority of fibroblast-like synoviocytes in the intimal lining layer expressed high levels of STAT1, whereas only limited expression of STAT1 was found in macrophages, regardless of their position in either intimal lining layer or synovial sublining. This is particularly interesting in light of the recent report by Hu et al, who demonstrated the existence of a delicate regulatory system for STAT1 in IFNγ signalling. They showed that exposure of macrophages to subthreshold concentrations of IFNγ sensitises these cells to subsequent IFNγ stimulation, which results in increased STAT1 expression. To find supporting evidence for the occurrence of this mechanism in vivo, they stated that STAT1 protein expression is increased in RA synovium, particularly in the intimal lining layer. In their rationale they implied that this might be due to intimal macrophages displaying increased STAT1 expression as a result of the IFNγ responses that lead to tissue abnormality in RA. However, our results demonstrate that the abundance of STAT1 in the intimal lining predominantly results from STAT1 expression by fibroblast-like synoviocytes.

It has been suggested that RA fibroblast-like synoviocytes not only respond to stimulation by proinflammatory cytokines but also show intrinsic molecular changes that are maintained in the absence of external stimuli. Here we demonstrated that increased expression of STAT1 in RA compared with OA fibroblast-like synoviocytes is sustained in culture. On the other hand, phosphorylation of STAT1 is almost completely lost in vitro, indicating that this is an active process that can be induced by cytokines in RA synovium. This notion is supported by the observation that STAT1 phosphorylation in fibroblast-like synoviocytes is rapidly restored upon stimulation with the type I interferon IFNβ. IFNβ is produced by fibroblast-like synoviocytes and abundant in synovium. Therefore, conceivably, this mechanism of STAT1 activation takes place in vivo in fibroblast-like synoviocytes. Type I interferons are believed to have immunosuppressive functions, including antiangiogenic effects. On the other hand, IFNβ produced by fibroblast-like synoviocytes can act as a survival factor for T cells in the rheumatoid joint. It is interesting to note that stimulation with IFNγ also induced STAT1 phosphorylation in fibroblast-like synoviocytes (data not shown). Because IFNγ expression is absent in the synovial lining this mechanism of STAT1 activation may rather be of importance in other synovial cell types.

Evidence for the importance of the Jak-STAT pathway in RA pathogenesis is accumulating, and has so far mainly been focused on the role of STAT3 and its growth promoting role. Accordingly, Shouda et al demonstrated that blockade of STAT3 by overexpression of SOCS3, a STAT3-induced inhibitor of Jak-STAT signalling, suppressed experimental arthritis. In contrast, an important effector function of STAT1 is its growth inhibitory effect. STAT1 is required for the growth restraint imposed by IFNz and IFNγ and the promotion of apoptosis. Krause et al showed that the proapoptotic action of STAT1 is antagonised by STAT3, which appears to be essential for RA synoviocyte survival. This is in line with the notion that STAT1 and STAT3 can serve as a tumour suppressor and a tumour promoter, respectively. Because our data demonstrate that besides the STAT3 signalling pathway the STAT1 pathway also is activated in fibroblast-like synoviocytes, it is tempting to speculate that the proapoptotic signals delivered by STAT1 in these cells in vivo are not strong enough to effectively counteract the STAT3 derived survival signals.

STAT1 may have a role as transcriptional activator as a STAT1 homodimer or as a constituent of a complex involving other factors, for example the STAT1/STAT2/IRF9 hetero-trimer. For the future it is challenging to know in which form STAT1 exerts its biological effects in ST, and what part activation of STAT1 plays in the fate of infiltrating lymphocytes. A better understanding of the balance of signals delivered through the different STAT pathways by dissecting the individual pathways and evaluating their contributions to either cell survival or growth arrest may
contribute to elucidating the disease mechanisms that are at play in RA.

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