Increased Synovial Tissue NF-κB1 Expression at Sites Adjacent to the Cartilage–Pannus Junction in Rheumatoid Arthritis

Maria J. Benito,1 Eithne Murphy,1 Evelyn P. Murphy,1 Wim B. van den Berg,2 Oliver FitzGerald,1 and Barry Bresnihan1

Objective. To compare the expression of the Rel/NF-κB subunits, NF-κB1 (p50) and RelA (p65), in paired synovial tissue samples selected from sites adjacent to and remote from the cartilage–pannus junction (CPJ) in patients with inflammatory arthritis.

Methods. Synovial tissue was selected at arthroscopy from sites adjacent to the CPJ and from the suprapatellar pouch of patients who were referred to an early arthritis clinic. Tissue samples from patients with osteoarthritis (OA) undergoing knee arthroplasty were also studied. Rel/NF-κB subunit activation and expression were measured by electrophoretic mobility shift assay and supershift analyses and by immunohistochemistry.

Results. Tissue samples were obtained from 10 patients with rheumatoid arthritis (RA), 7 with a seronegative arthropathy (SnA), and 6 with OA. Rel/NF-κB was abundantly expressed in all samples. In both RA and SnA synovial tissue, the absolute number of NF-κB1+ cells at the CPJ was significantly higher than at non-CPJ sites (P = 0.006 and P = 0.02, respectively). The proportion of cells expressing NF-κB1 was also significantly higher at the CPJ compared with non-CPJ sites (P = 0.003 in RA, P = 0.009 in SnA). The numbers of RelA+ cells were consistently lower throughout. In RA synovial tissue, but not in SnA synovial tissue, both the absolute number and the proportion of RelA+ cells were significantly higher at the CPJ than at non-CPJ sites (P = 0.003 and P = 0.01, respectively). In OA synovial tissue, the numbers of cells expressing NF-κB1 and RelA were similar to those observed at the non-CPJ sites in all inflammatory tissues studied.

Conclusion. In this study of early inflammatory arthritis, expression of NF-κB1 in synovial tissue was highest at sites most likely to be associated with joint erosion. These observations are consistent with a critical role of NF-κB1 in joint destruction, and support the rationale for specific therapeutic inhibition of NF-κB in RA.

Rheumatoid arthritis (RA) is characterized by prominent synovial tissue inflammation (1). Abundant new blood vessel formation enables infiltration of the proliferating synovial membrane by many activated cell populations that participate in complex pathophysiologic pathways. Previous studies of RA demonstrated increased macrophage accumulation at synovial tissue sites adjacent to the articular cartilage, in the cartilage–pannus junction (CPJ) (2). Moreover, lining layer macrophages at sites adjacent to the CPJ were found to express surface markers that suggested altered cell activation and differentiation (3). These observations imply that there are important phenotypic and functional differences between cell populations accumulating at sites of maximal joint damage and at sites more remote from the CPJ.

The Rel/NF-κB family of transcription factors is pivotal in the regulation of many genes, including cytokines, chemokines, and adhesion molecules, that participate in the pathophysiology of synovial inflammation and bone and cartilage degradation (4). Five mammalian Rel/NF-κB proteins have been described: NF-κB1 (p50, p105), NF-κB2 (p52, p100), RelA (p65), RelB, and...
c-Rel subunits. In unstimulated cells, the majority of the Rel/NF-κB subunits are retained in the cytoplasm through interaction with the inhibitory protein IκB. Cell stimulation induces degradation of IκB, resulting in nuclear translocation of the Rel/NF-κB subunits and activation of transcription. Activated Rel/NF-κB subunits are abundantly expressed in RA synovial tissue and in experimental models of arthritis (5–9). In studies involving experimental arthritis, there is evidence to suggest that the Rel/NF-κB subunits play distinct roles in the pathogenesis of inflammatory arthritis (10). Thus, in c-Rel–deficient mice, resistance to acute arthritis, but not to chronic destructive arthritis, was observed. In contrast, NF-κB1–deficient mice were found to be refractory to the induction of both acute and chronic destructive arthritis. These observations suggest that NF-κB1, but not c-Rel, is essential in the process of local joint inflammation and destruction.

The present study examined the hypothesis that NF-κB1 is the dominant Rel/NF-κB subunit at synovial tissue sites associated with maximal joint destruction. Synovial tissue samples were selected from areas adjacent to and remote from the CPJ in patients with early RA and in those with other types of inflammatory arthritis. Expression of the activated NF-κB1 and RelA subunits was observed in all patients. The predominant subunit expressed at the CPJ was NF-κB1. These findings are consistent with the suggestion from experimental studies that Rel/NF-κB subunits may have different roles in the pathogenesis of inflammatory arthritis, and that inhibition of NF-κB1 may modify the synovial tissue responses that lead to joint destruction.

PATIENTS AND METHODS

Patients. Patients with inflammatory polyarthritis were recruited to the study from the early arthritis clinic at St. Vincent’s University Hospital. A diagnosis of RA or psoriatic arthritis (PsA) was established according to widely accepted criteria (11,12). All clinical details were recorded by the same rheumatologist (EM) according to a predesigned protocol. Patients with inflammatory polyarthritis were recruited to the study from the early arthritis clinic at St. Vincent’s University Hospital. A diagnosis of RA or psoriatic arthritis (PsA) was established according to widely accepted criteria (11,12). All clinical details were recorded by the same rheumatologist (EM) according to a predesigned protocol. Patients were patients in accordance with a previously described method (13,16). The protein concentration was estimated with the Bradford protein assay kit (Bio-Rad, Richmond, CA). Prior to bandshift, the oligonucleotide probe was labeled with α32P-dCTP (Amersham, Braunschweig, Germany) using the Klenow enzyme (Promega, Madison, WI). The DNA–protein binding reaction was then conducted in a 20-μl reaction mixture, and 3 μg of nuclear protein extract was incubated for 20 minutes in the presence of 20 mM HEPES (pH 7.9), 5 mM MgCl2, 20% glycerol, 600 mM KCl, 0.2 mM EDTA, 8% Ficoll, 500 ng/μl polydeoxyinosinic-deoxycty tidyl acid (Sigma), 50 mM dithiothreitol, and α32P-dCTP–labeled double-stranded oligonucleotide probe containing the NF-κB binding site motif.
(5’-TGGGATTTTCCCATGAGTCT-3’). In some cases, non-labeled oligomer (100-fold excess) was also added as a cold competitor or a mutant sequence.

For supershift assays, 0.5 μg of antibodies specific to NF-κB1, NF-κB2, or RelA (Santa Cruz Biotechnology) were added following the initial 20-minute incubation, and were then coincubated for an additional 20 minutes at room temperature. The mixture was electrophoresed through a pre-run 5.5% native polyacrylamide in 0.5× Tris–borate–EDTA buffer. The loaded gel was run at 150 volts for 2 hours, dried, and autoradiographed on Kodak X-OMAT film (Eastman Kodak, Rochester, NY). The film was exposed overnight at 70°C and developed.

Statistical analysis. Analysis of the data was carried out to develop the descriptive statistics, and comparison of means was by independent t-test. Data are expressed as the mean ± SD, and P values less than 0.05 were considered significant, but were not corrected for the number of comparisons. Concordance of the 2 observations for each categorical variable was assessed by the kappa index. In order to evaluate the correlation between 2 measured values, a Pearson correlation was performed and the result is expressed as an r value.

RESULTS

Clinical and demographic details. Paired synovial tissue samples from 17 patients with inflammatory arthritis were studied. Symptoms of arthritis had been present for a mean 5.7 months (range 1–26 months) (Table 1). Ten of the patients had RA, and 7 had a seronegative arthropathy (SnA). Six of the patients with RA were female. Their ages ranged between 30 years and 65 years. Nine of the RA patients were rheumatoid factor (RF) positive; an RF test result was not available for 1 patient. Their C-reactive protein (CRP) levels were between 9 and 162 mg/liter. Of the 17 patients who were receiving a nonsteroidal antiinflammatory drug (NSAID) at the time of biopsy, 7 were in the RA group; only 1 patient with RA (patient 9) was receiving a disease-modifying antirheumatic drug (DMARD). None were receiving glucocorticoids.

Five of the 7 patients with SnA had a confirmed diagnosis of PsA. The ages of the SnA group ranged between 25 years and 59 years, and 4 were female. Their CRP levels ranged between 0 and 96 mg/liter. Six were receiving an NSAID and 1 (patient 15) was receiving a DMARD. For comparison, synovial tissue samples were obtained at knee arthroplasty from 6 patients with osteoarthritis (OA).

**NF-κB activation in synovial tissue.** To determine the levels of NF-κB binding activity in CPJ and non-CPJ synovial tissue, equal amounts of fresh nuclear extracts obtained from RA and SnA synovium were analyzed by EMSA using a labeled κB consensus sequence (Figure 1). Marked binding activity was detected in all samples from the 17 patients, indicating constitutive NF-κB binding activity in RA and SnA synovial tissue. For all tissue samples analyzed, the DNA–protein complexes detected corresponded to the NF-κB binding activity observed in IL-1β–stimulated RA synoviocytes (Figure 1). To further confirm the specificity of NF-κB

<table>
<thead>
<tr>
<th>Patient/sex/age, years</th>
<th>Diagnosis</th>
<th>Disease duration, months</th>
<th>RF titer</th>
<th>CRP, mg/liter</th>
<th>Swollen joint count</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/M/41</td>
<td>RA</td>
<td>2</td>
<td>1:160</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>2/M/47</td>
<td>RA</td>
<td>5</td>
<td>1:51,200</td>
<td>99</td>
<td>22</td>
</tr>
<tr>
<td>3/M/54</td>
<td>RA</td>
<td>26</td>
<td>1:2,560</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td>4/M/47</td>
<td>RA</td>
<td>6</td>
<td>1:640</td>
<td>30</td>
<td>3</td>
</tr>
<tr>
<td>5/F/39</td>
<td>RA</td>
<td>3</td>
<td>1:80</td>
<td>22</td>
<td>32</td>
</tr>
<tr>
<td>6/F/54</td>
<td>RA</td>
<td>2</td>
<td>1:1,280</td>
<td>74</td>
<td>21</td>
</tr>
<tr>
<td>7/F/57</td>
<td>RA</td>
<td>4</td>
<td>1:640</td>
<td>21</td>
<td>7</td>
</tr>
<tr>
<td>8/F/30</td>
<td>RA</td>
<td>1</td>
<td></td>
<td>NA</td>
<td>162</td>
</tr>
<tr>
<td>9/F/32</td>
<td>RA</td>
<td>4</td>
<td>1:160</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>10/F/65</td>
<td>RA</td>
<td>4</td>
<td>1:640</td>
<td>37</td>
<td>15</td>
</tr>
<tr>
<td>11/M/25</td>
<td>PsA</td>
<td>25</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12/M/59</td>
<td>PsA</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>13/F/36</td>
<td>PsA</td>
<td>2</td>
<td></td>
<td>96</td>
<td>23</td>
</tr>
<tr>
<td>14/F/44</td>
<td>PsA</td>
<td>8</td>
<td>0</td>
<td>79</td>
<td>3</td>
</tr>
<tr>
<td>15/F/59</td>
<td>PsA</td>
<td>11</td>
<td>0</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>16/F/38</td>
<td>UA</td>
<td>1</td>
<td>0</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>17/M/45</td>
<td>UA</td>
<td>3</td>
<td></td>
<td>1:160</td>
<td>58</td>
</tr>
</tbody>
</table>

* RF = rheumatoid factor; CRP = C-reactive protein; RA = rheumatoid arthritis; NA = not available; PsA = psoriatic arthritis; UA = undifferentiated arthritis.
subunit binding, cold competition with 100-fold molar excess of the \( \kappa B \) probe was performed.

The intensity of NF-\( \kappa B \) activation in each sample was quantified by densitometry. A wide range of NF-\( \kappa B \) activation was observed across the samples, and no significant differences between the 2 selected intraarticular sites or the disease categories were demonstrated (results not shown).

NF-\( \kappa B \) subunits in synovial tissue. To investigate which NF-\( \kappa B \) family members were responsible for the observed NF-\( \kappa B \) binding activity present in RA and SnA synovial tissue, nuclear extracts were preincubated with antibodies specific for NF-\( \kappa B1 \), NF-\( \kappa B2 \), and RelA subunits. Addition of either NF-\( \kappa B1 \) or RelA antiserum to nuclear extracts from CPJ and non-CPJ synovial tissues consistently resulted in both a prominent supershift and/or a reduction in the protein–DNA complex, whereas addition of NF-\( \kappa B2 \) antiserum did not alter the bandshift (Figure 2). Thus, only NF-\( \kappa B1 \) and RelA were selected for immunohistochemical analysis.

Abundant expression of the NF-\( \kappa B1 \) and RelA subunits was also demonstrated by immunohistochemistry in all of the synovial tissue samples examined (Figure 3). The NF-\( \kappa B1 \) subunit was present in endothelial cells, in many of the infiltrating perivascular cells, and in the cells accumulating in the synovial lining layer (Figures 3A and B). The RelA subunit was less widely expressed and present predominantly in endothelial cells, and only occasionally in sublining and lining layer cells (Figures 3C and D).

Localization of the NF-\( \kappa B \) subunits to the cell nuclei was clearly demonstrated by dual immunofluorescence (Figure 4). As shown in Figure 4A, most cells contained bright red–stained nuclei, representing the presence of activated nuclear NF-\( \kappa B1 \). There were a number of dull red–stained cells, representing nonactivated cytoplasmic NF-\( \kappa B1 \). Similarly, as shown in Figure 4B, activated RelA could be identified by the bright green–stained nuclei. The bright yellow–stained nuclei were an indication of the colocalization of activated nuclear NF-\( \kappa B1 \) and RelA (Figure 4C).

Infiltrating T lymphocyte and macrophage cell populations were quantified in the tissue samples selected from sites that were adjacent to the CPJ and remote from the CPJ. As expected, cellular infiltration was consistently greater at sites adjacent to the CPJ, where a strong correlation between the number of CD4+ and CD68+ cells was observed (\( r = 0.78 \)). A weaker correlation was observed at sites remote from the CPJ (\( r = 0.59 \)).

The number of NF-\( \kappa B1+ \) and RelA+ cells in tissue samples that were selected from sites adjacent to and distant from the CPJ was also compared (Figure 5). There were significantly more NF-\( \kappa B1+ \) cells adjacent to the CPJ, in both the RA and SnA synovial tissue (\( P = 0.006 \) and \( P = 0.02 \), respectively) (Figure 5a). In the OA tissue, the absolute number of NF-\( \kappa B1+ \) cells was

**Figure 1.** NF-\( \kappa B \) activation in synovial tissue from sites adjacent to and distant from the cartilage–pannus junction (CPJ). Total NF-\( \kappa B \) was quantified by electrophoretic mobility shift assay. The results from 1 patient with rheumatoid arthritis (patient 10 in Table 1) are demonstrated. Lanes 3 and 5 represent NF-\( \kappa B \) activation in the non-CPJ and CPJ tissue samples, respectively. Lanes 4 and 6 represent the competition assays, in which the reactions were performed with unlabeled probe. Positive and negative control experiments were performed in cultured synoviocytes by adding interleukin-1\( \alpha \) and 100-fold molar excess of the \( \kappa B \) probe was performed.

**Figure 2.** NF-\( \kappa B \) subunit expression in synovial tissue. Nuclear extracts were evaluated by supershift assay. Addition of either NF-\( \kappa B1 \) or RelA antiserum to nuclear extracts from cartilage–pannus (CPJ) and non-CPJ tissues from patients with rheumatoid arthritis (RA) and from patients with seronegative arthropathies consistently resulted in both a prominent supershift and/or a reduction in the protein–DNA complex, whereas addition of NF-\( \kappa B2 \) antiserum did not alter the bandshift. The results from 1 representative patient with RA (patient 10 in Table 1) are demonstrated. The bandshifts associated with anti-NF-\( \kappa B1 \), anti-NF-\( \kappa B2 \), and anti-RelA antibodies are demonstrated in lanes 3, 4, and 5, respectively. Lane 1 represents activation of total NF-\( \kappa B \). Lane 2 shows the negative control obtained by addition of 100-fold molar excess of unlabeled probe.
similar to the numbers at the non-CPJ sites of the inflammatory tissues.

Further analysis demonstrated that the proportions of infiltrating cells that expressed the NF-κB1

Figure 3. Immunohistochemical analysis of NF-κB subunit expression in synovial tissue. Tissue was selected from sites adjacent to (A and C) and distant from (B and D) the cartilage–pannus junction, and stained with anti–NF-κB1 (A and B) and anti-RelA (C and D) (original magnification × 200). NF-κB1 and RelA expression is shown in greater detail in E and F, respectively (original magnification × 1,000).

Figure 4. Dual immunofluorescence analysis of NF-κB subunit expression in synovial tissue. Nuclear localization of NF-κB1 in red (A) and Rel-A in green (B), as well as colocalization of NF-κB1 and Rel-A in yellow (C), is demonstrated (original magnification × 200). In A, most cells contain bright red–stained nuclei, representing the presence of activated nuclear NF-κB1. In B, activated RelA can be identified by the bright green–stained nuclei. In C, the bright yellow–stained nuclei represent colocalization of nuclear NF-κB1 and RelA. The dull red–, green–, and yellow–stained cells represent nonactivated cytoplasmic NF-κB subunit expression.

Figure 5. Quantification of NF-κB subunit expression in synovial tissue. NF-κB1+ (a) and Rel-A+ (b) cells in tissue sections selected from sites adjacent to and distant from the cartilage–pannus junction (CPJ and NCPJ, respectively) of patients with rheumatoid arthritis (RA), psoriatic arthritis (PsA), and osteoarthritis (OA) were quantified by immunohistochemistry.
ures 3C and D). There were significantly more RelA+ cells adjacent to the CPJ in RA tissue ($P = 0.003$), but not in SnA tissue (Figure 5b). The number of RelA+ cells in OA synovial tissue was similar to the numbers at sites distant from the CPJ in all inflammatory arthritis tissues.

The proportions of cells that expressed RelA were significantly higher at sites adjacent to the CPJ, in both the RA and SnA synovial tissue ($P = 0.01$ and $P = 0.02$, respectively) (results not shown). Thus, in RA tissue, between 1.5% and 6.5% of cells adjacent to the CPJ expressed RelA at these sites, compared with between 1.3% and 2.3% of cells at sites distant from the CPJ. Similarly, in SnA tissue, between 2.5% and 5.0% of the cells at sites adjacent to the CPJ expressed RelA, compared with between 0.3% and 1.2% of cells at sites distant from the CPJ.

**DISCUSSION**

The principal finding in this study was the increased expression of 2 Rel/NF-κB subunits, NF-κB1 and RelA, in synovial tissue samples selected from sites adjacent to the CPJ in patients with RA. Increased Rel/NF-κB expression at the CPJ was due not only to increased cellular infiltration, but also to an increased proportion of cells at the CPJ that expressed Rel/NF-κB subunits. The predominant subunit expressed at the CPJ, which is the site of joint erosion in RA, was NF-κB1. This finding is consistent with the observations in experimental studies which have suggested that the Rel/NF-κB subunits may have different roles in the pathogenesis of RA (10). Thus, NF-κB1, but not c-Rel, is essential for the induction of both local joint inflammation and destruction.

The patients with RA selected for this study had relatively early disease, with a mean duration of symptoms of <6 months. All patients had active disease. In the majority of patients with RA, radiographic evidence of joint damage begins to appear during the first 6–12 months after the onset of disease, and continues to progress for many years (17,18). Critical components of the pathophysiologic pathways associated with articular matrix degradation can be identified in the synovial tissue of patients with RA very early in the course of the disease (19–21). Macrophage, T lymphocyte, synoviocyte, and endothelial cell nuclei may express activated Rel/NF-κB following IL-1β or tumor necrosis factor α (TNFα) stimulation (4). Cell proliferation, angiogenesis, leukocyte trafficking, the production of cytokines and other mediators of inflammation and joint damage, and dysregulation of apoptosis are dependent on Rel/NF-κB activation. These mechanisms are activated in RA, SnA, and OA (22–24). Joint erosions appear in both RA and SnA, and it is likely that NF-κB plays a similar critical role in the proinflammatory and degradative pathways associated with each category of arthritis.

The tissue samples included in this study were selected under direct vision from sites that were as close as possible to the CPJ, and from sites within the same knee joint, including the suprapatellar pouch, that were remote from the CPJ. EMSA was used to examine the DNA-binding properties of NF-κB in RA synovial tissue. Similar binding activities were demonstrated in tissue samples from CPJ sites and those from non-CPJ sites. Quantification of Rel/NF-κB subunit expression is not feasible with the use of supershift assays. The immunohistologic method utilized in this study to quantify mononuclear cell populations in synovial tissue has been validated in several previous studies (2,3,14,20). The observed increase in NF-κB1 expression at sites adjacent to the CPJ is consistent with the increased levels of macrophage infiltration and activation that have been previously described at the CPJ (2,3).

Thirteen of the 17 patients with inflammatory arthritis who were included in this study were receiving NSAIDs at the time of biopsy. Sodium salicylate and aspirin may inhibit NF-κB activation by preventing the degradation of IκB (25). In addition, NSAIDs can effectively block NF-κB–induced gene expression by inhibiting IκB degradation (26,27). However, it is unlikely that treatment with NSAIDs influenced the conclusions from this study, because the effects of NSAIDs on NF-κB expression would have been similar at the 2 tissue sites that were evaluated.

Therapeutic strategies that have targeted individual pathophysiologic mediators, such as TNFα and IL-1, have produced significant clinical benefits for many, but not all, patients with RA (28–31). Improved strategies may evolve from the combined targeting of more than one critical mediator of inflammation and destruction, or from upstream targeting at pivotal transcriptional levels. Previous studies have demonstrated that Rel/NF-κB blockade may modulate inflammatory mechanisms in the synovium (32). The predominant expression of NF-κB1 at tissue sites adjacent to the CPJ is consistent with the suggestion from experimental studies that specific Rel/NF-κB subunits may play distinct pathophysiologic roles in chronic arthritis (10). Thus, our results support the rationale for specific therapeutic inhibition of NF-κB1 in RA.
REFERENCES


