Immunohistochemical analysis of rheumatoid synovial tissue: methodologic and pathogenetic studies

Smeets, T.J.M.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
THE EFFECTS OF INTERFERON-β TREATMENT ON SYNVOIAL INFLAMMATION AND EXPRESSION OF METALLOPROTEINASES IN PATIENTS WITH RHEUMATOID ARTHRITIS.

Tom J. M. Smeets 1, Jean M. Dayer 2, Maarten C. Kraan 1, Johannes Versendaal 3, Rachel Chicheportiche 2, Ferdinand C. Breedveld 3, and Paul P. Tak 1.

1 Division of Clinical Immunology and Rheumatology, Academic Medical Center, Amsterdam, The Netherlands
2 Division of Immunology and Allergy, Department of Internal Medicine, University Hospital, Geneva, Switzerland
3 Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands

Arthritis & Rheumatism 2000;43:270-4
ABSTRACT

Objective. Interferon-β (IFNβ) treatment is emerging as a potentially effective form of therapy in various immune-mediated conditions. This study evaluated the effects of IFNβ therapy on the cell infiltrate, cytokine profile, and expression of metalloproteinase 1 (MMP-1) in synovial tissue from patients with rheumatoid arthritis (RA). To further assess the mechanism of action, in vitro experiments were conducted to determine the effects of IFNβ on the production of MMP-1, MMP-3, tissue inhibitor of metalloproteinases 1 (TIMP-1), and prostaglandin E₂ (PGE₂) by human fibroblast-like synoviocytes (FLS).

Methods. Eleven patients were treated for 12 weeks with purified natural fibroblast IFNβ (Frone, Ares-Serono, Geneva, Switzerland) subcutaneously 3 times weekly with the following dosages: 6 million IU (n=4), 12 million IU (n=3), and 18 million IU (n=4). Synovial biopsy specimens were obtained by needle arthroscopy at 3 time-points: directly before and at 1 month and 3 months after initiation of treatment. Immunohistologic analysis was performed using monoclonal antibodies specific for the following phenotypic markers and mediators of joint inflammation and destruction: CD3, CD38, CD68, CD55, tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), IL-6, MMP-1, and TIMP-1. In addition, we measured the production of MMP-1, MMP-3, TIMP-1, and PGE₂ by RA FLS and dermal fibroblasts in the presence and absence of IFNβ.

Results. A statistically significant reduction in the mean immunohistologic scores for CD3+ T cells and expression of MMP-1, and TIMP-1 at 1 month, CD38+ plasma cells and expression of IL-6 at 3 months, and expression of IL-1β at both 1 and 3 months was observed in synovial tissue after IFNβ treatment. The scores for CD68+ macrophages and TNFα expression also tended to decrease, but the differences did not reach statistical significance. The in vitro experiments revealed inhibition of MMP-1, MMP-3, and PGE₂ production by RA FLS, whereas TIMP-1 production was only slightly decreased. These effects were more consistent in RA FLS than in dermal fibroblasts.

Conclusion. The changes in synovial tissue after IFNβ treatment and the in vitro data support the view that IFNβ therapy has immunomodulating effects on rheumatoid synovium and might help to diminish both joint inflammation and destruction. Larger well-controlled studies are warranted to show the efficacy of IFNβ treatment for RA.

INTRODUCTION

Interferon-β (IFNβ) treatment is emerging as a potentially effective form of therapy in various immune-mediated conditions. IFNβ therapy might also be an alternative approach for the treatment of rheumatoid arthritis (RA) patients. This type I interferon could inhibit tumor necrosis factor (TNFα) and interleukin-1β (IL-1β) secretion, and enhance IL-10 and IL-1 receptor antagonist production (for review, see refs.1 and 2). Conceivably, concurrent targeting of TNFα, IL-1β, and other proinflammatory cytokines by use of counterregulatory cytokines, such as IFNβ, could be more effective in suppressing joint destruction than inhibition of TNFα alone (3). Other possible immunomodulatory effects of IFNβ treatment include enhancement of T cell cytotoxicity, regulation of antibody production, inhibition of T cell proliferation and migration, enhancement of IL-2 production by Th1 cells, inhibition of IFN-γ production by activated peripheral blood mononuclear cells (PBMC), up-regulation of transforming growth
factor β1 (TGFβ-1) and TGFβ receptor type II expression on PBMC, down-regulation of class II major histocompatibility complex (MHC) expression on monocytes, enhancement of class I MHC expression on virus-infected cells and tumor cells, activation of natural killer cells, down-regulation of several adhesion molecules, and enhancement of soluble adhesion molecules in serum (1,2,4).

IFNβ treatment of patients with relapsing remitting multiple sclerosis reduces the rate of exacerbation and decreases the number and frequency of lesions on magnetic resonance images. Recent studies showed that IFNβ therapy also ameliorates collagen-induced arthritides (CIA) in rodents and primates. Constitutive expression of IFNβ by gene therapy resulted in reduced paw swelling, improvement in histologic features, decreased serum levels of anticollagen antibodies, and suppression of cytokine production in DBA/1 mice with CIA (5). A study in rhesus monkeys with CIA revealed remarkable clinical improvement and decreased serum levels of C-reactive protein after systemic treatment with recombinant IFNβ1a (1). A pilot study in 12 RA patients showed statistically significant improvement after 3 months of treatment (1).

The aim of the present study was to evaluate the effects of IFNβ therapy on the cell infiltrate, cytokine profile, and the expression of metalloproteinase 1 (MMP-1) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in the synovial tissue from RA patients who were treated with IFNβ. To further assess the mechanism of action, in vitro experiments were conducted in the presence and absence of IFNβ, determining its effect on the production of MMP-1, MMP-3, TIMP-1, and prostaglandin E2 (PGE2) by RA fibroblast-like synoviocytes (FLS) and dermal fibroblasts.

PATIENTS AND METHODS

Patients

Eleven patients who participated in a pilot study of the effects of IFNβ in RA (1) were evaluated. All patients fulfilled the American College of Rheumatology (ACR; formerly the American Rheumatism Association) criteria for RA (6) and had active RA, defined as: ≥ 6 tender joints, ≥ 6 swollen joints, and at least 1 of the following 2 criteria: duration of morning stiffness ≥ 45 min, or an erythrocyte sedimentation rate ≥ than 28 mm/hr. Nine patients were taking disease-modifying antirheumatic drugs, the dosage of which has been stable for at least 3 months prior to study entry and during the study. All patients gave informed consent, and the study protocol was approved by the Medical Ethics Committee of the Leiden University Medical Center.

The patients were treated for 12 weeks with purified natural fibroblast IFN-β (Frone, Ares-Serono, Geneva, Switzerland), which was injected subcutaneously 3 times weekly as follows: patient 1-4 received 6 million IU, patients 5-7 received 12 million IU, and patients 8-11 received 18 million IU. Clinical assessment, performed by 1 observer (PPT), included tender joint count (68 joints), swollen joint count (66 joints), patient’s global assessment of pain (on a 0-10-cm visual analogue scale (VAS)), patient’s global assessment (on a 0-10-cm VAS), and physician’s global assessment (on a 0-10-cm VAS). Laboratory analysis included serum levels of C-reactive protein. Patients were considered to have responded to treatment if they fulfilled at least 20 % improvement in the tender joint count and the swollen joint count and 20 % improvement in at least 3 of the remaining clinical and laboratory assessments as described above (7).
Chapter 9

Synovial tissue
Small-bore arthroscopy (2.7-mm arthroscope, Storz, Tuttingen, Germany) was performed under local anesthesia. Biopsies of synovial tissue were obtained from the entire joint using a 2-mm grasping forceps (Storz) at 3 time points: before, 1 month after, and 3 months after treatment. On each occasion, an average of at least 20 biopsy samples was obtained. Biopsy specimens were not available from patient 11 at 3 month after initiation of treatment. The tissue was collected and snap-frozen en bloc in Tissue-Tek OCT (Miles, Elkhart, IN) by immersion in methybutane (-70°C). Frozen blocks were stored in liquid nitrogen until sectioned for staining. Five-micrometer sections were cut in a cryostat and mounted on glass slides (Star Frost adhesive slides, Knittelgläser, Germany); slides were stored at -70°C until immunohistochemical analysis could be performed.

Immunohistochemistry
Serial sections were stained with the following mouse monoclonal antibodies (Mab): anti-CD3 (Becton Dickinson, San Jose, CA) to detect T cells, anti-CD38 (Becton Dickinson) to detect plasma cells, anti-CD68 (EBM11, DAKO, Glostrup, Denmark) to detect macrophages, Mab 67, which recognizes CD55 on FLS, anti-MMP-1 (36665.111, R&D Systems Europe, Abingdon, UK), and anti-TIMP-1 (7-6C1, Oncogene Research Products, Cambridge, MA). Staining was also done with the following rabbit polyclonal antibodies: anti-TNFα (IP-300, Genzyme, Cambridge, MA), anti-IL-1β (LP-712, Genzyme), and anti-IL-6 (Dept. of Nephrology, Leiden University Medical Center, Leiden, The Netherlands). For control sections, the primary antibodies were omitted or irrelevant antibodies were applied.

Staining for cell markers was performed, as described previously (8). Following a primary step of incubation with Mab, bound antibody was detected according to a 3-step immunoperoxidase method. Staining for MMP-1 and TIMP-1 was performed using biotinylated tyramine for amplification. Horseradish peroxidase activity was detected using hydrogen peroxide as substrate and amino ethylcarbazole as dye. Alkaline phosphatase-conjugated swine anti-rabbit antibodies (DAKO), naphtol-AS-MX-phosphate, Fast Red Violet LB and levamisol (Sigma, St. Louis, MO) were used to detect the rabbit polyclonal antibodies, as described previously (8).

Microscopic analysis
All sections were coded and randomly analyzed. After immunohistochemical staining, sections were scored semiquantitatively on a 5-point scale by 2 independent observers (TJMS and JV), who were unaware of the clinical data, as described previously (8,9). CD68 expression was scored separately in the intimal lining layer and the synovial sublining. Minor differences between the observers were resolved by mutual agreement.

In vitro experiments on fibroblast-like synoviocytes and dermal fibroblasts
FLS from RA patients and dermal fibroblasts were prepared as described previously (10). Synovial biopsy specimens were obtained from patients other than those described in the clinical study. The experiments were performed on 2 separate preparations of both FLS and dermal fibroblasts. Cells at passage 3 were seeded at 20,000 cells/well and incubated for 48 h in DMEM with 10% fetal calf serum followed by another 48 hours in the presence or absence of 125 pg/ml of IL-1β together with various concentrations of IFNβ. Thereafter medium was removed and stored at −20°C, as previously described (10). Medium was analyzed for levels of MMP-1.
Synovial Tissue Responses to Interferon-β

(Bindazyme pro-MMP-1 enzyme immunoassay kit, The Binding Site, Birmingham, UK), MMP-3 (Bindazyme pro-MMP-3 enzyme immunoassay kit, The Binding Site), TIMP-1 (TIMP-1 human ELISA system, Biotrak, Amersham International, Little Chalfont, UK), and PGE₂(10).

Statistical analysis
The Wilcoxon signed ranks test for matched pairs was used to compare data before and after treatment. Bonferroni corrections for multiple comparisons were not applied in this exploratory study.

RESULTS

Clinical and demographic features
The clinical study group consisted of 6 women and 5 men. The mean (± SD) age was 49 ± 15 years (range 27 to 68 years), and the mean duration of disease was 50 ± 38 months (range 2 to 111 months). Erosions were present in 8 of the 11 patients and 9 patients were seropositive for IgM rheumatoid factor. The study patients exhibited on average significant improvement in the tender joint count, swollen joint count, patient’s assessment of pain, patient’s global assessment, and physician’s global assessment after 3 months of IFNβ therapy (all P < 0.05), as described previously (1). Patients 1 and 4 fulfilled the ACR criteria for 20% improvement (20% ACR response) at 1 month after initiation of IFNβ administration and patients 1, 4, 6, and 7 had a 20% ACR response at 3 months after initiation of IFNβ.

Findings of immunohistochemistry
The negative control sections were all negative. The tissue from patient 9 at month 1 could not be evaluated. In all cases where data were missing because the tissue sections did not fulfill our quality control criteria to allow reliable evaluation, the patients were not significantly different from other patients participating in the study. The mean scores for cell markers, cytokines, MMP-1, and TIMP-1 are shown in Table 1. In the patients treated with 6 million IU, 12 million IU, and 18 million IU of IFNβ, nonparametric analysis revealed a significant decrease in the mean scores for infiltration by CD3 + T cells (from 2.3 ± 1.1 to 1.6 ± 0.8; P = 0.03), and for expression of MMP-1 (from 2.6 ± 1.0 to 1.6 ± 0.8; P=0.02) and TIMP-1 (from 2.6 ± 1.2 to 1.8 ± 1.3; P=0.03) at month 1, but not at month 3 (Figure 1). There was also a significant decrease in the scores for IL-1β at month 1 (from 2.6 ± 1.1 to 1.6 ± 0.8; P = 0.02) and at month 3 (from 2.6 ± 1.1 to 1.9 ± 1.0; P = 0.03). The scores for infiltration by CD38 + plasma cells (from 2.1 ± 1.0 to 1.5 ± 1.2; P = 0.04) and expression of IL-6 (from 1.9 ± 0.8 to 1.1 ± 0.7; P = 0.04) were reduced at month 3. Changes in the synovial features were also observed in synovial tissue from patients who had a modest clinical response, but who did not fulfill the 20% ACR criteria. The changes in scores 1 month after initiation of IFNβ treatment seemed to be more pronounced than the changes in scores at month 3 after initiation of IFNβ treatment. The scores for CD68+ macrophages in the synovial sublining and TNFα expression also tended to decrease, but these differences did not reach statistical significance. There was no clear relationship between the effects on the synovium and the dosage of IFNβ (data not shown); patients were treated with presumably therapeutic dosages in all groups.
### Table I. Mean semiquantitative scores for the expression of CD3+ T-cells, CD38+ plasma cells, CD68+ macrophages, CD55+ fibroblast-like synoviocytes, and for the expression of IL-1β, TNFα, IL-6, MMP-1, and TIMP-1 in synovial tissue before, after one month, and after 3 months of IFNβ therapy. Values are the mean ± SD.

<table>
<thead>
<tr>
<th></th>
<th>Before therapy</th>
<th>After 1 month of therapy</th>
<th>After 3 months of therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>2.3 ± 1.1</td>
<td>1.6 ± 0.8 (1)</td>
<td>1.9 ± 1.4</td>
</tr>
<tr>
<td>CD38</td>
<td>2.1 ± 1.0</td>
<td>1.4 ± 0.9 (1)</td>
<td>1.5 ± 1.2 (1)</td>
</tr>
<tr>
<td>CD68 (lining)</td>
<td>1.7 ± 0.9</td>
<td>1.2 ± 0.4 (1)</td>
<td>1.7 ± 0.7</td>
</tr>
<tr>
<td>CD68 (sublining)</td>
<td>2.6 ± 1.3</td>
<td>2.3 ± 1.2</td>
<td>2.2 ± 1.0</td>
</tr>
<tr>
<td>CD55</td>
<td>1.1 ± 0.5</td>
<td>1.0 ± 0.8</td>
<td>1.2 ± 0.6</td>
</tr>
<tr>
<td>IL-1β</td>
<td>2.6 ± 1.1</td>
<td>1.6 ± 0.8 (1)</td>
<td>1.9 ± 1.0 (1)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>2.6 ± 1.1</td>
<td>2.0 ± 0.7</td>
<td>1.9 ± 0.9</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.9 ± 0.8</td>
<td>1.4 ± 1.2</td>
<td>1.1 ± 0.7 (1)</td>
</tr>
<tr>
<td>MMP-1</td>
<td>2.6 ± 1.0</td>
<td>1.6 ± 0.8 (1)</td>
<td>2.2 ± 1.4</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>2.6 ± 1.2</td>
<td>1.8 ± 1.3 (1)</td>
<td>2.3 ± 1.3</td>
</tr>
</tbody>
</table>

**Findings of in vitro experiments on FLS and dermal fibroblasts**

In vitro experiments revealed that IFNβ inhibits the production of MMP-1 and MMP-3 by RA FLS both in the presence and in the absence of IL-1β (Table 2). TIMP-1 production was only slightly decreased by IFNβ in the presence of IL-1β. PGE2 production was diminished by IFNβ in the presence and in the absence of IL-1β.

In contrast, there was no clear effect of IFNβ on MMP-1, MMP-3, and TIMP-1 production by dermal fibroblasts. However, PGE2 production was inhibited, showing that the cells responded to IL-1β and IFNβ. The results were similar for the 2 separate populations of FLS and dermal fibroblasts that were investigated.
DISCUSSION

The results presented in this study demonstrate changes in the synovium during IFNβ therapy of RA patients. A statistically significant reduction in the mean immunohistologic scores for infiltration by CD3+ T-cells and CD38+ plasma cells, as well as reduced scores for the expression of IL-1β, IL-6, MMP-1, and TIMP-1, was observed in synovial biopsy specimens from RA patients after IFNβ therapy. There was also a tendency towards lower scores for infiltration by CD68+ macrophages and TNFα expression after therapy. In vitro experiments revealed that IFNβ can decrease the production of MMP-1, MMP-3, and PGE2 FLS from RA patients.

<table>
<thead>
<tr>
<th>Cells</th>
<th>IFN-β U/ml</th>
<th>MMP-1 (μg/ml)</th>
<th>MMP-3 (μg/ml)</th>
<th>TIMP-1 (μg/ml)</th>
<th>PGE2 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLS</td>
<td>0</td>
<td>2.07 ± 0.12</td>
<td>2.754 ± 0.15</td>
<td>0.55 ± 0.03</td>
<td>2.12 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>10^-5</td>
<td>0.95 ± 0.05</td>
<td>1.45 ± 0.22</td>
<td>0.24 ± 0.05</td>
<td>0.88 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>10^-4</td>
<td>0.51 ± 0.06</td>
<td>1.00 ± 0.17</td>
<td>0.13 ± 0.03</td>
<td>0.69 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>10^-3</td>
<td>0.43 ± 0.02</td>
<td>1.03 ± 0.09</td>
<td>0.11 ± 0.01</td>
<td>0.35 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>10^-2</td>
<td>0.50 ± 0.07</td>
<td>0.83 ± 0.19</td>
<td>0.14 ± 0.02</td>
<td>0.66 ± 0.13</td>
</tr>
<tr>
<td>Dermal fibroblasts</td>
<td>0</td>
<td>0.23 ± 0.02</td>
<td>1.5 ± 0.10</td>
<td>0.90 ± 0.27</td>
<td>2.61 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>10^-7</td>
<td>0.32 ± 0.02</td>
<td>2.06 ± 0.26</td>
<td>1.05 ± 0.01</td>
<td>3.93 ± 0.69</td>
</tr>
<tr>
<td></td>
<td>10^-4</td>
<td>0.44 ± 0.13</td>
<td>1.65 ± 0.13</td>
<td>1.15 ± 0.36</td>
<td>3.32 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>10^-3</td>
<td>0.52 ± 0.08</td>
<td>1.80 ± 0.25</td>
<td>1.05 ± 0.30</td>
<td>2.68 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>10^-2</td>
<td>1.13 ± 0.04</td>
<td>2.2 ± 0.35</td>
<td>2.07 ± 0.44</td>
<td>2.75 ± 0.35</td>
</tr>
</tbody>
</table>

Table 2. Effects of IFNβ on the production of MMP-1, MMP-3, TIMP-1 and PGE2 by rheumatoid FLS and dermal fibroblasts in the presence and absence of IL-1β (125 pg/ml). Values are the mean ± SEM of 3 experiments. Similar results were obtained in a separate experiment using different preparations of FLS and dermal fibroblasts.

Semiquantitative analysis is a reliable and cheap method that allows evaluation of the entire synovial tissue sections in a time-efficient manner. Highly significant correlations can be found with manual counting and digital image analysis (9). The changes described in the present study are substantial in light of the conservative microscopic scoring system that we used (9). Although it seems unlikely that the features of synovial inflammation are susceptible to placebo effects, this cannot be completely excluded until placebo-controlled studies become available. This is the first study to describe changes in the cell infiltrate and cytokine expression in synovial tissue from RA patients following IFNβ treatment. Our findings confirm and extend the observations in the joints of mice with CIA after IFNβ gene therapy (5). The reduction in the number of T-cells could be explained by an effect on T-cell trafficking secondary to
downregulation of adhesion molecules by IFNβ (11). Inhibition of T-cell proliferation (12) may also play a role. The reduction in the number of plasma cells in RA synovial tissue suggests that IFNβ could also affect humoral immune responses. This notion is supported by previous studies, showing that IFN-β inhibits T-cell-dependent immunoglobulin secretion in vitro (13) and the anticollagen antibody response in mice with CIA in vivo (5).

Figure 1. Mean (+SEM) changes in the semiquantitative scores for infiltration by CD3+ T cells, CD38+ plasma cells, CD68+ macrophages, CD55+ fibroblast-like synoviocytes and for the expression of IL-1β, TNFα, IL-6, MMP-1, and TIMP-1 in synovial biopsies at 1 month (■) and at 3 months (▲) after initiation of IFNβ therapy. *=P<0.05.

The clinical improvement after IFNβ therapy (1) was associated with a reduction in the cell infiltrate, as well as with diminished expression of IL-1β and MMP-1, which are believed to play a major role in joint destruction. Since TIMP-1 expression is also reduced after treatment, it is at present unclear whether changes in the balance between MMPs and TIMPs after IFNβ therapy occur and whether they would alter the course of joint destruction. However, our in vitro data showing that IFNβ has a marked inhibitory effect on MMP-1, MMP-3, and PGE₂ production, rather than on TIMP-1 production, by RA FLS provide an additional argument for a possible role of IFNβ in preventing joint destruction.

The data from this study suggest that some of the changes in the synovial tissue could be transient. Similarly, a recent study in patients with multiple sclerosis showed a significant decrease in the number of circulating T-cells expressing HLA-DR antigens and CD25 after 2 months of treatment, followed by a return to pretreatment levels after 3 - 12 months (14). This might be caused by down-regulation of IFNβ receptors or the development of neutralizing antibodies to IFNβ during treatment (15). The return to pretreatment values was, however, not associated with reduced clinical efficacy. It is at present unclear whether the clinical effects in RA patients will continue during prolonged IFNβ therapy.

Taken together, the changes in synovial biopsy specimens from RA patients treated with IFNβ and the in vitro effects on FLS support the view that IFNβ therapy has immunomodulating effects on rheumatoid synovium and might have a beneficial effect on both joint inflammation and erosive disease. The results warrant larger well-controlled studies.
References


3. van den Berg WB. Joint inflammation and cartilage destruction may occur uncoupled. Springer Semin Immunopathol 1998;20:149-64.


