Fatal attraction: chemokines and rheumatoid arthritis
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SYNOVIAL TISSUE MACROPHAGES: A SENSITIVE BIOMARKER FOR RESPONSE TO TREATMENT IN RHEUMATOID ARTHRITIS PATIENTS

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ABSTRACT

Objective. Previous work identified synovial sublining macrophage numbers as a potential biomarker for clinical efficacy in rheumatoid arthritis (RA). Therefore, we investigated the association between changes in synovial macrophages infiltration and clinical improvement after antirheumatic treatment.

Methods. 88 patients who participated in various clinical trials were studied. All patients underwent serial arthroscopy before initiation of treatment and after different time intervals. Immunohistochemical and digital image analysis was performed according to standardized procedures to detect changes in CD68+ synovial sublining macrophages in relationship to changes in the Disease Activity Score (DAS28). Statistical analysis was performed using one-way ANOVA, the independent samples t-test, linear regression, and the standardized response mean (SRM).

Results. For the different groups according to the DAS28 response criteria (good, moderate, and non-responders) there was a statistically significant difference in the change in sublining macrophages (mean cells/mm² ± SEM: -643 ± 124, -270 ± 64, and -95 ± 60, respectively; P<0.0003). There was a significant correlation between the change in the number of macrophages and the change in DAS28 (Pearson correlation 0.874, P < 0.01). The change in sublining macrophages could explain 76% of the variation in the change in DAS28 (P < 0.02). The sensitivity to change of the biomarker was high in actively treated patients (SRM > 0.8) while the ability to detect changes in placebo treated patients was weak (SRM <0.3).

Conclusion. The results suggest that changes in synovial sublining macrophages can be used to predict possible efficacy of antirheumatic treatment.

INTRODUCTION

The recent increase in the development of a variety of new, targeted therapies clearly raises the need for sensitive biomarkers, which could be used for selection purposes during the development process. The acquisition of optimal tissue samples has been greatly enhanced by technological developments in needle arthroscopy (1). Reliable microscopic quantification of synovial inflammation has been facilitated by advances in computerized image analysis. By these means, sequential synovial biopsies have recently been analyzed in several clinical trials evaluating the effects of disease modifying antirheumatic drugs (DMARDs), biologic therapies, and targeted small molecules. These studies suggested consistent associations between the rapidity and magnitude of both the clinical and immunohistologic responses. No consensus however has previously emerged as to the optimal markers in tissues that are representative of disease activity or sensitive to change.

A prior cross-sectional study in 62 RA patients using stepwise multiple regression analysis revealed that scores for local disease activity are particularly associated with the number of macrophages in the synovial sublining as well as the expression of macrophage-derived cytokines (2). Recently, we conducted a randomized trial to determine the question which feature in RA synovial tissue (ST) samples could be used as a biomarker for clinical efficacy in relatively small studies of short duration (3). Patients received either prednisolone according to the COBRA regimen (4) or placebo for 2 weeks. ST samples were obtained before initiation of treatment and at 2 weeks. Twenty-four immunohistologic markers were investigated in this study. Each of the endpoints was statistically analyzed using an analysis model of covariance (ANCOVA). The model fitted included terms for treatment as a fixed effect and the baseline measurement as a covariate. The aim was to assess the treatment
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difference. This study identified sublining macrophages as the best biomarker associated with the clinical response to corticosteroids (3).
The utility of CD68+ macrophages in the sublining layer as a candidate biomarker now requires to be tested across discrete interventions and kinetics. The objective of the present study was to investigate the changes in this biomarker after different therapies and after different time intervals in relationship to the clinical response to treatment to validate the analysis of synovial macrophages in clinical studies.

PATIENTS AND METHODS

Patients.
ST was obtained by arthroscopy under local anesthesia at 2 time points for each patient from a clinically active wrist, knee, or ankle joint of 88 patients who fulfilled the ACR criteria for RA (5). Before each arthroscopy the Disease Activity Score (DAS28) was calculated (6). All patients had active disease before commencing treatment, measured by a tender and swollen joint count of at least six joints in combination with a raised ESR and/or CRP and/or significant morning stiffness. All patients were included in clinical trials and the complete patient group comprised 7 subgroups receiving distinct treatment regimens.

Fifteen patients who were DMARD naïve for at least 28 days started with methotrexate (MTX) treatment (7). Treatment was started with 7.5 mg/week and increased stepwise to 15.0 mg/week in 12 weeks. Synovial biopsies were taken before initiation of treatment and after 112 days of treatment from the same joint.

Fifteen patients who were DMARD naïve for at least 28 days started with leflunomide (LEF) treatment after the first arthroscopy (7). Treatment was started with a loading dose of 100 mg/day during the first 3 days; patients received 20 mg/day thereafter. Synovial biopsies were obtained before initiation of treatment and after 112 days of treatment from the same joint.

Ten patients who were on stable DMARD treatment (MTX, sulphasalazine, hydroxychloroquine, LEF, or a combination of these drugs) for at least 28 days were given 2-week oral treatment of prednisolone at 60mg/day for 7 days followed by 40mg/day for another 7 days (3). Before treatment and at 2 weeks synovial biopsies were taken from the same joint.

Infliximab treatment was started in 20 patients. These patients were on stable MTX for at least 28 days prior to infliximab treatment and stable prednisone was allowed at ≤ 10 mg/day. Patients were dosed with 3 mg/kg infliximab on day 1 (directly after the first arthroscopy) and on day 15 (8). Arthroscopies were performed before initiation of treatment and on average after 28 days.

Ten active RA patients who were on stable DMARD therapy for at least 28 days were treated with a specific CCR1 antagonist (9). Patients were allowed to use stable low dose prednisone ≤ 10 mg/day. They underwent arthroscopy directly before and after 14 days of treatment.

Some of the above mentioned clinical trials were placebo-controlled studies (3;8;9). Therefore this allowed us to include a control group of 18 patients who underwent serial synovial biopsies with different intervals while they were on stable DMARD treatment (mostly MTX). These patients received a placebo treatment as detailed in the referred studies.

Arthroscopy.
Synovial tissue was obtained by arthroscopy under local anesthesia in all patients at baseline (before treatment) and directly after completion of each treatment strategy. Arthroscopies.
tissue sampling, and storage were performed according to standardized procedures, as described previously in detail (7).

**Immunohistochemistry**

Synovial tissue sections were stained with a monoclonal mouse-anti-human anti-CD68 antibody (EBM11, Dako, Glostrup, Denmark). Sections with non-assessable tissue, defined by the absence of an intimal lining layer, were omitted before analysis. For control sections, the primary antibodies were omitted or irrelevant isotype-matched mouse antibodies were applied. Staining was performed according to a 3-step immunoperoxidase method as previously described in detail (10).

**Digital image analysis.**

Stained slides were evaluated by computer-assisted image analysis. All sections were coded and analyzed in a random order by an independent observer who was blinded for the clinical data, as described previously (11). For each patient the change in the number of total positive cells per square mm ST was calculated for sublining macrophages, but intimal macrophages were also evaluated.

**Statistical analysis.**

Mean differences between independent groups were determined using the one-way ANOVA and the independent-samples t-test. Significant differences were confirmed by non-parametric tests. The association between the clinical measurements and the immunohistochemical results was quantified using Pearson’s correlation coefficient. The immunohistochemical factors with significant correlation coefficients (P < 0.05) were entered into a linear regression analysis, testing for variables capable of explaining clinical response. The immunohistochemical markers were assigned as the independent variable. The probability of a significant association between the independent and dependent variables was defined as P < 0.05.

Standardized response means (SRMs) of the clinical scores and immunohistochemical scores were calculated to evaluate the ability of these measurements to detect changes over time in the different study groups. The SRM is the mean change in a score in a defined period of time divided by the standard deviation of the change in that score. An SRM of >0.8 is considered high, representing a change of at least four-fifths of a standard deviation of the change in the score. An SRM of 0.5 is defined as having a moderate potential to detect changes, and an SRM of 0.2 as having low potential (12).

**RESULTS**

**Clinical features.**

In total 88 patients were included and analyzed in this study. Baseline disease activity and treatment characteristics are depicted in Table 1.

All patients had active disease at baseline measured by the DAS28 (6.02 ± 0.11 [mean ± SEM]). On average there were no differences in age, gender and disease duration between the different subgroups, for further demographic and clinical characteristics we refer to the published data of all subgroups (3-7-9).

The 70 patients who started on active treatment showed on average a significant change in the DAS28 of -1.37 ± 0.14 (mean ± SEM) after treatment (P < 0.001), whereas the DAS28 remained the same in the 18 control patients (change in DAS28: -0.13 ± 0.12) (Table 2).
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Table 1. Treatment characteristics and baseline disease activity.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Active/Control group</th>
<th>Number of patients</th>
<th>Interval (days)</th>
<th>DAS²₈ baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methotrexate</td>
<td>Active</td>
<td>15</td>
<td>112</td>
<td>6.15 [4.50-7.83]</td>
</tr>
<tr>
<td>Leflunomide</td>
<td>Active</td>
<td>15</td>
<td>112</td>
<td>6.05 [4.38-7.31]</td>
</tr>
<tr>
<td>Prednisone</td>
<td>Active</td>
<td>10</td>
<td>14</td>
<td>6.26 [4.58-7.98]</td>
</tr>
<tr>
<td>Infliximab</td>
<td>Active</td>
<td>20</td>
<td>27</td>
<td>6.07 [4.14-8.21]</td>
</tr>
<tr>
<td>CCR1 antagonist</td>
<td>Active</td>
<td>10</td>
<td>14</td>
<td>5.84 [4.31-6.95]</td>
</tr>
<tr>
<td>Stable MTX</td>
<td>Control</td>
<td>6</td>
<td>2</td>
<td>5.97 [4.22-8.21]</td>
</tr>
<tr>
<td>Stable DMARD</td>
<td>Control</td>
<td>12</td>
<td>14</td>
<td>5.69 [3.43-7.59]</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td>88</td>
<td>53 [5]</td>
<td>6.02 [0.11]</td>
</tr>
</tbody>
</table>

The number of good, moderate and non-responders was calculated for all patients according to the DAS28 response criteria (13). In total 11 patients fulfilled the criteria for good responders (i.e. improvement > 1.2 and DAS28 at endpoint ≤ 3.2), 35 patients were moderate responders (i.e. improvement >0.6 and ≤ 1.2 and DAS28 at endpoint ≤ 5.1, or improvement > 1.2 and DAS28 at endpoint > 3.2) and 42 patients were considered non-responders.

Changes in sublining macrophages are associated with clinical improvement. The changes in macrophage numbers in the intimal lining layer and sublining as well as the percentual changes in sublining macrophages compared to baseline, in relationship to changes in the DAS28 are depicted in Table 2 for the various trials. There was a statistically significant difference in the mean change in the number of sublining macrophages between good responders (-643 ± 124), moderate responders (-270 ± 64), and non-responders (-95 ± 60) (one-way ANOVA: P < 0.0003; between group differences were determined by t-tests and confirmed by non-parametric tests). The intimal macrophages tended to be decreased in all groups. There were no significant differences in the mean change from baseline between the 3 response groups for the number of intimal macrophages (Figure 1).

Of interest, the mean change in the number of sublining macrophages per substudy was highly correlated to the mean change in DAS28, according to the two-tailed Pearson correlation coefficient (Pearson correlation 0.874, P < 0.01) (Figure 2). There were no correlations between the change in intimal macrophages and the change in DAS28 (data not shown). Linear regression analysis, weighted for the number of patients in each substudy, revealed that the mean change in sublining macrophages could significantly explain 76 percent of the variance in the mean change in DAS28 grouped per substudy (P < 0.02).
Table 2. Mean change in the number of intimal lining CD68+ macrophages (± s.e.m.), mean change in the number of sublining CD68+ macrophages (± s.e.m.), the mean percentual change in sublining CD68+ macrophages and the mean change in DAS28 (± s.e.m.) compared to baseline for each sub study.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean change in the number of intimal lining CD68+ macrophages (s.e.m.)</th>
<th>Mean change in the number of sublining CD68+ macrophages (s.e.m.)</th>
<th>Mean percentual change for sublining CD68+ macrophages</th>
<th>Mean change in DAS28 (s.e.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prednisolone start [10;14]</td>
<td>-35 (46)</td>
<td>-492 (89)</td>
<td>-51 %</td>
<td>-2.15 (0.40)</td>
</tr>
<tr>
<td>Remicade [20;28]</td>
<td>-100 (30)</td>
<td>-275 (84)</td>
<td>-47 %</td>
<td>-1.39 (0.28)</td>
</tr>
<tr>
<td>Leflunomide start [15;112]</td>
<td>-28 (17)</td>
<td>-286 (112)</td>
<td>-38 %</td>
<td>-1.29 (0.31)</td>
</tr>
<tr>
<td>Methotrexate start [15;112]</td>
<td>-44 (17)</td>
<td>-292 (106)</td>
<td>-41 %</td>
<td>-1.36 (0.25)</td>
</tr>
<tr>
<td>CCR1 antagonist [10; 14]</td>
<td>-192 (81)</td>
<td>-338 (131)</td>
<td>-44 %</td>
<td>-0.72 (0.27)</td>
</tr>
<tr>
<td>DMARD stable [12;14]</td>
<td>40 (38)</td>
<td>152 (107)</td>
<td>+ 13 %</td>
<td>-0.23 (0.10)</td>
</tr>
<tr>
<td>Methotrexate stable [6;2]</td>
<td>-71 (29)</td>
<td>26 (158)</td>
<td>+ 22 %</td>
<td>0.07 (0.28)</td>
</tr>
<tr>
<td>Active treatment grouped</td>
<td>-76 (17)</td>
<td>-321 (46)</td>
<td>-44 %</td>
<td>-1.37 (0.14)</td>
</tr>
<tr>
<td>[70, 53]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls grouped</td>
<td>3 (29)</td>
<td>110 (87)</td>
<td>+17 %</td>
<td>-0.13 (0.12)</td>
</tr>
<tr>
<td>[18, 10]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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Figure 1. Mean values of the change compared to baseline in the number of CD68+ macrophages in the intimal lining layer (A.) and synovial sublining (B.) for respectively non-responders, moderate responders and good responders according to the DAS28 response criteria in the total study population.

Figure 2. Correlation between the mean change per sub study in the number of CD68+ sublining macrophages and the mean change in DAS28 (p<0.01 Pearson correlation 0.874, weighted linear regression p<0.02, Rsquare = 0.755, 95% confidence interval (95% CI) 0.001-0.005 (t = interval between first and second measurement, n = number of patients, TI = treatment initiation).
Changes in sublining macrophages may predict active treatment.

To determine the sensitivity to change, SRMs of the changes after treatment were calculated. As shown in Figure 3 the SRMs in individual substudies were high for both sublining macrophages and DAS28 after active treatment. When patients from all active treatment substudies were grouped (n=70), the SRMs of the change in both the DAS28 and sublining macrophages were high (1.16 and 0.83, respectively). In the control group where the second biopsy was performed after two days (n=6) the SRM for both DAS28 and sublining macrophages was about zero (0.10 and 0.07, respectively). In the control group where the second biopsy was performed after two weeks (n=12), the SRM for the DAS28 was -0.64 and the SRM for sublining macrophages 0.40. When both control groups were grouped the SRM for the change in DAS28 was -0.23 and the SRM for the change in sublining macrophages was 0.30, consistent with the notion that the biological marker is less susceptible to placebo effects or expectation bias than clinical evaluation.

Figure 3. Standardized Response Mean (SRM) of the change in DAS28 (SRM DAS28) and the number of CD68+ macrophages in the sublining (SRM CD68sI) for the individual studies (t= interval between first and second measurement, n= number of patients, TI = treatment initiation) and grouped for patients who received initiation of an active compound (n=70) and placebo (n=18) (the solid line indicates the 0.5 SRM cut off (moderate), the dotted line indicates the 0.8 SRM cut off (high)).

DISCUSSION

The results of this study show that changes in numbers of synovial sublining macrophages are correlated to clinical improvement independent of the therapeutic strategy. Furthermore, this study demonstrates that the change in the numbers of sublining macrophages may be used to explain clinical outcome. Of importance, the data indicate that the change in the number of sublining macrophages could be used as a sensitive biomarker to predict possible efficacy of novel antirheumatic treatment.

Previous work suggested an association between the number of synovial macrophages and joint destruction in RA (14). Moreover, analysis of the synovial cell infiltrate revealed a positive correlation between scores for local disease activity and the number of macrophages.
as well as expression of macrophage-derived cytokines (TNF-α and IL-6) in rheumatoid ST, suggesting that macrophage numbers are associated with clinical signs of inflammation (2). In keeping with this concept, macrophage numbers are increased in clinically involved joints compared to clinically uninvolved joints of RA patients (15). It appears likely that various pathogenetic mechanisms might lead to a final common pathway resulting in synovial macrophage activation. This may cause increased production of a variety of pro-inflammatory cytokines and chemokines involved in the development of symptoms like pain and swelling (16-18). The importance of macrophage-derived cytokines for the clinical expression of the disease is underscored by the beneficial effect of strategies aimed at targeting TNF-α, IL-1, and IL-6 (19-21). Of note, by far most of the macrophages in actively inflamed joints are localized in the synovial sublining rather than in the intimal lining layer (22).

The results presented here are consistent with previous studies examining the effects of treatment with DMARDs and biological agents at the site of inflammation. It has been shown that various effective agents like gold, sulphasalazine, methotrexate, and leflunomide can reduce macrophage infiltration RA ST (7:23-25). Moreover, a predominant reduction in macrophage numbers of the synovial membrane has been reported in patients who achieved clinical remission, as defined by the ACR criteria, induced by use of various DMARDs (26). Similarly, treatment with anti-TNF-α antibodies and the IL-1 receptor antagonist resulted in a reduction of macrophage numbers (8:27:28). The same results were obtained using a specific CCR1 antagonist (9).

It should be noted that inflammatory cells other than macrophages may be reduced as well after antirheumatic treatment, depending on the specific mechanism of action and the duration of treatment. Obviously, these specific cell types and their mediators of inflammation also need to be evaluated in studies focused on the mechanism of action of targeted therapies. The immunohistologic parameters may be correlated to each other to a certain extent. Recently, we conducted a study to provide a greater understanding of the changes in the ST alongside clinical response by using a known clinically effective therapy, prednisolone (3). The ANCOVA model showed that clinically effective prednisolone therapy was particularly associated with a marked reduction in macrophage infiltration in the RA ST after 2 weeks of treatment. Comparable results were obtained after infliximab treatment (8).

Based on these observations, we investigated whether this biomarker might exhibit similar changes after different therapeutic regimens and after varying duration of treatment. The results of the present study show that this is the case. There is a highly significant correlation between changes in sublining macrophages and clinical improvement, independent of the studied specific therapies. The changes may be observed as early as 14 days after initiation of effective treatment, but treatment for more prolonged periods results in a more pronounced decrease in macrophage infiltration. Patients who are good, moderate, or non-responders according to the DAS28 response criteria differ significantly with regard to changes in the number of sublining macrophages. Additionally, it is possible to explain the change in DAS28 based upon the change in sublining macrophages, which implies a direct relationship between macrophages and clinical measures of disease activity.

Moreover, we investigated the sensitivity to change of this biomarker after active treatment or placebo. According to the SRM the sensitivity to change after active treatment is good for both DAS28 and sublining macrophages. The SRMs calculated for changes in DAS28 and sublining macrophages after placebo treatment suggest that the biological marker may be less susceptible to placebo effects or expectation bias than clinical evaluation. This might be explained by the subjective components included in clinical measures of disease activity (29).

This notion is supported by a previous study in an independent patient cohort using semiquantitative analysis, showing unaltered immunohistologic scores in serial synovial biopsies obtained after placebo treatment (30). In conclusion, the results of this study indicate
that synovial sublining macrophages might be used as a biomarker for the evaluation of novel antirheumatic therapies. In addition to providing insight into the mechanism of action of treatment, this approach may help to screen for possible efficacy.

Reference List


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