Targeted therapies in rheumatoid arthritis
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CHAPTER 4

Rituximab abrogates joint destruction in rheumatoid arthritis by inhibiting osteoclastogenesis

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ABSTRACT

Objectives: To examine how rituximab may result in inhibition of joint destruction in rheumatoid arthritis (RA) patients.

Methods: Twenty-eight patients with active RA were treated with rituximab. Radiographs of hands and feet before and 1 year after therapy were assessed using the Sharp-van der Heijde score (SHS). Expression of bone destruction markers was evaluated by immunohistochemistry and immunofluorescence of synovial biopsies obtained before and 16 weeks after initiation of treatment. Serum levels of osteoprotegerin (OPG), receptor activator of nuclear factor-kappaB ligand (RANKL), osteocalcin and cross-linked N-telopeptides of type I collagen (NTx) were measured by ELISA before and 16 weeks post-treatment.

Results: After 1 year, the mean (SD) change in total SHS was 1.4 (10.0). Sixteen weeks after treatment there was a decrease of 99% in RANK-positive osteoclast precursors ($P = 0.02$) and a decrease of 37% ($P = 0.016$) in RANKL expression in the synovium and a trend towards reduced synovial OPG expression (25%, $P = 0.07$). In serum, both OPG (20%, $P = 0.001$) and RANKL (40%, $P = <0.0001$) levels were significantly reduced 16 weeks after treatment, but the OPG/RANKL ratio increased (157%, $P = 0.006$). We found a trend towards an increase of osteocalcin levels ($P = 0.053$), while NTx concentrations did not change.

Conclusions: Rituximab treatment is associated with a decrease in synovial osteoclast precursors and RANKL expression and an increase in the OPG/RANKL ratio in serum. These observations may in part explain the protective effect of rituximab on progression of joint destruction in RA.
INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammation of the synovial tissue and destruction of the underlying cartilage and bone, both resulting in functional impairment. The goal of antirheumatic treatment is not only to attenuate the clinical symptoms of joint inflammation, but also to inhibit the progression of joint destruction.1

The presence of rheumatoid factors (RF) and/or anti-citrullinated protein antibodies (ACPA), produced by B lineage cells, is a predictor of radiological damage.2-5 Rituximab, an anti-CD20 directed B cell depletive therapy, is an effective treatment for RA patients, especially for those positive for rheumatoid factor and/or ACPA.6-10 Importantly, rituximab treatment also reduces the progression of joint destruction in RA patients, even in the absence of a clear-cut clinical response.11,12 Taken together, these data suggest an association between B cells and the development of joint destruction.

Insight into the interplay between immune cells and cells involved in bone remodelling in RA is evolving. In chronic arthritis, resorption of mineralised cartilage and subchondral bone is mediated by osteoclasts, which are present in inflamed synovial tissue of RA patients at the area of bone erosions.13 Arthritis can be induced in mice lacking osteoclasts, but these mice do not develop bone erosions14,15, illustrating the critical role of osteoclasts in arthritic bone loss. Receptor activator of nuclear factor-kappaB (RANK), a member of the TNF receptor family, and its ligand (RANKL) are important stimulators of osteoclast differentiation and activation, and inhibitors of osteoclast apoptosis. The RANK-RANKL interaction is inhibited by osteoprotegerin (OPG), a naturally occurring, soluble decoy receptor for RANKL.16

Proinflammatory cytokines important in RA pathogenesis, such as TNF-α, interleukin (IL)-1 and IL-6, not only stimulate production of both RANKL and OPG, but also act directly on osteoclasts and their precursors.17 Furthermore, matrix metalloproteinases (MMPs), expressed by fibroblast-like synoviocytes (FLS), macrophages, neutrophils and chondrocytes, have a key role in degradation of unmineralised cartilage and their production is also augmented by these cytokines (reviewed in 18).

The cellular and molecular mechanisms involved in rituximab’s effect on joint erosions are as yet unknown. Exploration of the ongoing processes in serum and synovium, the target tissue of RA, before and after B cell depletion, could teach us more about the cross-talk between the immune system and the cells involved in bone resorption in RA. Therefore, we examined the effects of B cell depletion on the RANK/RANKL/OPG system.

PATIENTS AND METHODS

Patients. Twenty-eight RA patients were included from an open-label study on the synovial tissue response to rituximab treatment that was previously reported.19 All patients were diagnosed according to the 1987 ACR classification criteria for RA20 and had active disease despite methotrexate treatment. Active RA was defined as having ≥4 tender joints and ≥4 swollen joints of 28 joints assessed, and at least one of the following: erythrocyte sedimentation rate (ESR) ≥28 mm/h, serum C-reactive protein (CRP) levels ≥15 mg/l or morning stiffness ≥45 minutes. The study was performed according to the Declaration of Helsinki and approved by the Medical Ethics Committee of the Academic Medical Center (AMC)/University of Amsterdam; all patients gave written informed consent.
Study design. All patients were treated with two intravenous infusions of 1,000 mg rituximab (day 1 and 15), as previously described. Premedication with methylprednisolone was omitted to study the specific effect of rituximab. The disease activity score in 28 joints (DAS28) was measured before and every month after treatment. Serum and synovial tissue were obtained before (i.e., baseline) and 16 weeks after initiation of treatment.

Serum enzyme-linked immunosorbent assays. Serum levels of OPG were measured before and 16 weeks after treatment as part of a multiplex enzyme-linked immunosorbent assay (ELISA), as previously described. Serum levels of total soluble RANKL (sRANKL; Immunodiagnostik, Bensheim, Germany), osteocalcin (Immunodiagnostic Systems, Liege, Belgium) and NTx (Osteomark®, Inverness Medical, Princeton, New Jersey) were measured by ELISA before and after initiation of treatment.

Synovial biopsy, immunohistochemical analysis and quantification of stained tissue sections. At baseline and at week 16, serial synovial biopsies were collected by needle arthroscopy of the same actively inflamed joint, as previously described. Frozen sections (5 μm) were stained with the following mouse monoclonal antibodies: anti-OPG (clone 98A1071, Imgenex, San Diego, CA), anti-RANK (clone 80707, R&D Systems, Minneapolis, MN) to detect osteoclast precursors and anti-tartrate-resistant acid phosphatase-1 (TRAcP-1; clone ZY-9CS, Invitrogen, Breda, the Netherlands) to detect mature osteoclasts. Staining of TRAcP-1 was performed using a three-step immunoperoxidase method, as previously described. For staining of RANK, OPG and all other cellular markers, bound antibody was detected with a polymer-horseradish peroxidase anti-mouse antibody (Envision+ System, Dako, Glostrup, Denmark) and aminoethylcarbazole (AEC; Dako) was used as dye. For the TRAcP staining, sections from an osteoblastoma containing numerous mature osteoclasts (obtained from the Pathology department of the AMC) were used as a positive control. As a negative control, irrelevant/isotype-matched immunoglobulins were applied to the sections instead of the primary antibody or the primary antibody was omitted.

The expression of synovial markers was quantified using digital image analysis, as previously described. The expression level of RANK-positive cells was presented as cell counts/mm²; OPG was presented as integrated optical density (IOD)/mm², an arbitrary unit representing the intensity of staining per mm².

Immunofluorescence to detect RANKL in synovial tissue. Rabbit-anti-human RANKL (AbCam, Cambridge, UK) was developed with donkey anti-rabbit Rhodamine (Jackson ImmunoResearch). Sections were firstly adsorbed with PBS supplemented with 2% bovine serum albumin for 10 mins. Primary and secondary antibodies diluted in PBS supplemented with 2% bovine serum albumin were incubated at room temperature in the dark for 1 hr or 30 minutes respectively. Sections were immersed in Hoechst 33258 (Sigma) at 20μg/ml for 2 minutes for nuclear counterstaining, then mounted and kept in the dark at -20°C. A Zeiss LSM 780 Zen confocal microscope was used to visualise staining and Zeiss LSM Image Examiner software was used for pixel quantification. The expression level of RANKL was presented as pixels/μm².

Radiographic assessments. Radiographs of hands and feet were obtained at baseline and at different time points after the initiation of rituximab treatment. The same observer, who was
blinded to radiograph sequence, evaluated paired radiographs using the Sharp-van der Heijde scoring method (SHS), which consists of the joint space narrowing score and erosion score (range 0-448). Using an assumption of linear progression, the change in SHS after 1 year was calculated.

**Statistical analysis.** Continuous data were described as mean and standard deviation (SD), if normally distributed, and as median and range, if not normally distributed. Correlations were calculated using Spearman’s rho. The Wilcoxon signed-rank test for paired data was used to analyse the change after treatment of the different parameters analysed in synovial tissue and serum. All statistical analyses were performed with SPSS 17.0 software (SPSS, Chicago, IL). A P-value of ≤ 0.05 was considered statistically significant.

**RESULTS**

**Clinical characteristics.** The demographic and clinical features of the cohort are shown in Table 1. Of note, this population had a long disease duration and had failed previous treatment.

**Table 1. Baseline characteristics of patients**

<table>
<thead>
<tr>
<th>Demographics</th>
<th>(n = 28)</th>
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<tbody>
<tr>
<td>Female, n (%)</td>
<td>23 (82%)</td>
</tr>
<tr>
<td>Age (years)*</td>
<td>55 (22-75)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Baseline disease status</th>
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<tbody>
<tr>
<td>IgM-RF positive, n (%)</td>
</tr>
<tr>
<td>ACPA positive, n (%)</td>
</tr>
<tr>
<td>DAS28*</td>
</tr>
<tr>
<td>ESR (mm/hr)*</td>
</tr>
<tr>
<td>CRP (mg/l)*</td>
</tr>
<tr>
<td>Disease duration (years)*</td>
</tr>
<tr>
<td>Nodular disease, n (%)</td>
</tr>
<tr>
<td>Erosive disease, n (%)</td>
</tr>
<tr>
<td>Total SHS*</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Medication</th>
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</thead>
<tbody>
<tr>
<td>Concomitant methotrexate, n (%)</td>
</tr>
<tr>
<td>Concomitant methotrexate dosage (mg/wk)*</td>
</tr>
<tr>
<td>Concomitant oral prednisone, n (%)</td>
</tr>
<tr>
<td>Concomitant oral prednisone dosage (mg/day)*</td>
</tr>
<tr>
<td>No. of previous DMARDs*</td>
</tr>
<tr>
<td>No. of previous biologicals*</td>
</tr>
</tbody>
</table>

Data are presented as n (%), median (range)* or mean (standard deviation [SD])* as appropriate. Erosive disease is defined as erosions described by radiologist together with rheumatologist. IgM-RF = IgM rheumatoid factor; ACPA = anti-citrullinated peptide antibodies; DAS28 = disease activity score in 28 joints; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; SHS = Sharp-van der Heijde score; DMARDs = disease-modifying antirheumatic drugs.
with several disease-modifying antirheumatic drugs (DMARDs) and biologicals. Sixteen weeks post-treatment there was a mean (SD) decrease in DAS28 of 1.6 (1.1), and 2 patients (7%) had a good response, 18 a moderate response (64%) and 8 no response (29%) according to the European League Against Rheumatism (EULAR) response criteria.27

**Radiographic outcomes.** First, we analysed the progression in joint destruction after 1 year. From 25 of the 28 patients paired radiographs of hands and feet were available from before and after rituximab treatment. The median (range) of the total SHS score was 36 (0-247) at baseline and 39 (0-246) 1 year post-rituximab. After 1 year, the mean (SD) change in total SHS was 1.4 (10.0), change in joint space narrowing score was 0.6 (5.0) and change in erosion score 0.8 (5.0) (Figure 1A). Of the 25 patients analysed, 7 patients (28%) showed progression of joint destruction of more than one point in the total SHS and only 4 patients (16%) increased more than three points in 1 year (Figure 1B). The three patients without any erosion at baseline stayed non-erosive after 1 year. Of note, these data are comparable to the mean changes in radiological scores that were seen in a large clinical trial evaluating the effect of rituximab in RA patients with a disease duration of more than 10 years.11 Although in that clinical trial a different scoring system for erosive destruction was used (total Genant-modified Sharp score), it supports the notion that our cohort is representative.

![Figure 1. Radiographic outcomes 1 year after treatment with rituximab. Mean (SD) changes after 1 year of the total Sharp-van der Heijde score (SHS), joint space narrowing (JSN) score and erosion score of 25 rheumatoid arthritis patients (A). Individual changes of the total SHS after 1 year (B).](image)

**Effect of rituximab on synovial osteoclast precursors, osteoprotegerin, RANKL expression and osteoclasts.** For the synovial tissue analysis of RANK- and OPG expression, we included paired biopsies of 20 patients, based on availability and fulfilment of quality control criteria. Consistent with previous observations from our group 28, only seven patients (35%) showed RANK expression and nine patients showed OPG expression (45%) at baseline. There was no correlation between baseline expression of RANK or OPG on the one hand, and total SHS on the other (r = -0.22, P = 0.39 and r = -0.18, P = 0.49, respectively). After treatment with rituximab, there was a significant reduction of 99% in RANK-positive osteoclast precursors at week 16 (P = 0.02), while OPG expression showed a non-significant decrease (25%, P = 0.07; Figure 2). To detect the expression of RANKL, we used immunofluorescence and included paired biopsy samples.
of 11 patients, for whom sufficient material was present to perform this analysis. All patients expressed RANKL at baseline and the expression tended to correlate with the total SHS score \((r = 0.60, P = 0.05)\) at baseline. After treatment with rituximab, we observed a significant decrease of 37% of RANKL expression \((P = 0.016, \text{Figure 2})\). No TRACP-positive mature osteoclasts could be detected in any of the synovial tissue samples (data not shown). Separate analyses showed that changes in synovial bone and cartilage destruction markers were not related to changes in SHS or disease activity (data not shown).

![Figure 2](image)

**Figure 2.** Expression of RANK, OPG and RANKL in synovium before and 16 weeks after treatment with rituximab. Individual changes in synovial osteoprotegerin (OPG; A), synovial receptor activator of nuclear factor-kappaB (RANK; B) expression and its ligand (RANKL; C) of 20 (A and B) or 11 (C) rheumatoid arthritis patients. Treatment effect was compared using a Wilcoxon signed-rank test; \(^*P < 0.05\). Representative examples of immunohistochemical and immunofluorescence staining of synovium for OPG before (A1) and after (A2) treatment, for RANK before (B1) and after (B2) treatment and for RANKL before (C1) and after (C2) treatment with rituximab. Magnification 20x (A and B) and 10x (C).

Changes in serum levels of osteoprotegerin, sRANKL and the OPG/sRANKL ratio after rituximab treatment. As we saw a strong effect of rituximab on the RANK/RANKL/OPG system in the synovial tissue, we also analysed OPG and sRANKL levels in serum of 28 patients. Sixteen weeks after initiation of rituximab treatment, we found a statistically significant decrease in serum levels of both OPG and sRANKL (from 3253 (median) to 2796 pg/ml, 20%, \(P = 0.001\) and from 30.2 to 16.5 ng/ml, 40%, \(P = <0.0001\), respectively). Of importance, the OPG/sRANKL ratio
increased (from 0.06 (median) to 0.09, 157%, \( P = 0.006 \); Figure 3). Separate analyses showed that changes in serum bone and cartilage destruction markers were not related to changes in SHS or disease activity (data not shown).

**Figure 3.** Serum levels of sRANKL and OPG, and the OPG/sRANKL ratio before and 16 weeks after treatment with rituximab. Individual changes in soluble receptor activator of nuclear factor-kappaB ligand (sRANKL; A) and serum osteoprotegerin (OPG; B) expression, and the OPG/sRANKL ratio (C) of 28 rheumatoid arthritis patients. Treatment effect was compared using a Wilcoxon signed-rank test; \( **P < 0.01 \).

**Change in soluble markers of bone turnover.** Finally, we studied the effect of rituximab on systemic biomarkers of bone turnover. We measured serum osteocalcin levels as a marker of bone formation and levels of cross-linked N-telopeptides of type I collagen (NTX) as a biomarker of bone resorption before and 16 weeks after initiation of rituximab treatment. We found a strong trend towards an increase of osteocalcin levels (from (median) 12 to 12.5, \( P = 0.053 \)), while NTX concentrations did not change (Figure 4).

**DISCUSSION**
These results show that rituximab treatment strongly affects the RANK/RANKL/OPG system in synovium and peripheral blood of patients with active RA. The number of RANK-positive osteoclast precursors in synovial tissue was decreased by 99% and RANKL expression was decreased by 37%, while synovial OPG showed a non-significant reduction. In serum, however, both sRANKL and OPG decreased, whereas the OPG/sRANKL ratio was elevated. These alterations in the RANK/RANKL/OPG system were not related to radiological progression, as assessed by the SHS. However, joint destruction was stabilised in a large majority of patients, indicating that rituximab’s interference with the mediators of osteoclastogenesis resulted in
inhibition of further bone loss. This is in line with a large, randomised clinical trial in which rituximab inhibited the progression of joint destruction in the majority of patients with RA, including those who did not experience an ACR response.\textsuperscript{11} Taken together, these data provide at least in part an explanation for the effective inhibition of joint destruction observed in clinical trials after B cell depletive therapy.

What could be the exact role of B cells in rheumatic joint destruction? It is generally known that RANKL is produced by FLS\textsuperscript{29} and T cells\textsuperscript{10} in a membranous or soluble (sRANKL) form after stimulation by IL-1, TNF-α and IL-17, a product of T helper 17 (T\(_{\text{H17}}\)) cells.\textsuperscript{31,32} However, B cells are also able to secrete RANKL.\textsuperscript{33,34} Of importance, a recent study using cytokine mRNA profiling identified B cells as major producers of RANKL in synovial fluid of RA patients\textsuperscript{35}. Proinflammatory cytokines also stimulate secretion of OPG by FLS and dendritic cells in synovium, as well as CD40L on T cells. T cell activation is an important function of B cells in synovium of RA patients.\textsuperscript{36} Hence, B cells may also upregulate both RANKL and OPG production by stimulating T cells. We have previously shown in the same cohort that rituximab induces a significant albeit variable decrease in synovial B cells and an indirect decrease in T cells, plasma cells, macrophages and lymphoid neogenesis 16 weeks after initiation of rituximab treatment.\textsuperscript{19} Thus, the observed changes in the RANK/RANKL/OPG system may at least in part be explained by decreased cell infiltration.

RANK is primarily expressed on mononuclear macrophage-lineage cells, including pre-osteoclastic cells, dendritic cells, fibroblasts, and B and T cells\textsuperscript{37} which are abundantly present in inflamed synovial tissue. RANKL interacts with RANK via direct cell-cell contact and thereby promotes differentiation of osteoclast precursors into mature osteoclasts.\textsuperscript{31} RANK-positive osteoclast precursors were seen in 35% of our patients, but TracP-positive mature osteoclasts could not be detected in synovium. This is in contrast with several other studies, which showed osteoclasts to be present at the interface of inflamed synovial tissue and the periosteal surface (reviewed in\textsuperscript{38}). These differences might be explained by the use of synovial tissue obtained during joint replacement surgery in these previous investigations versus arthroscopy-guided synovial biopsies in the present study. Direct physical contact with the skeletal matrix is

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{Serum levels of osteocalcin and NTx before and 16 weeks after initiation of treatment with rituximab. Individual changes in osteocalcin (A) and cross-linked N-telopeptides of type I collagen (NTx; B) of 28 rheumatoid arthritis patients. Treatment effect was compared using a Wilcoxon signed-rank test. nM BCE = nmol Bone Collagen Equivalents per liter}
\end{figure}
required for the differentiation into mature osteoclasts \(^3\), but arthroscopic synovial biopsies do not include the junction between this periosteal surface and synovium. Nevertheless, we were able to analyse changes in the interaction between immune cells and bone resorptive precursor cells at the site of inflammation, as described above.

A recently published, small study investigated whether rituximab treatment had a systemic effect on bone remodelling in 13 active RA patients resistant to TNF-\(\alpha\) antagonist therapy; synovial tissue was not assessed. There was a trend towards reduced sRANKL levels in the serum which did not reach statistical significance, perhaps due to the small number of patients. Of interest, levels of the bone resorption marker desoxypyridinoline were significantly reduced after rituximab treatment. \(^4\)

Other antirheumatic therapies have been reported to have various effects on the RANK/ RANKL/OPG system in the synovium.\(^41\)-\(^43\) Treatment with TNF-\(\alpha\) antagonists, which has a clear inhibitory effect on the progression of joint destruction even in the absence of a clinical response,\(^44\) for 12 weeks induced an increase in synovial OPG expression without affecting RANKL, resulting in an increased OPG/RANKL ratio.\(^41\) Similarly, successful treatment with different DMARDs other than TNF-\(\alpha\) inhibitors led to increased OPG and decreased RANKL expression and an increased OPG/RANKL ratio.\(^42\) In contrast, clinical responders to anakinra (IL-1 receptor antagonist), administered as monotherapy or in combination with pegsenercept (PEGylated soluble TNF receptor type I, an TNF-\(\alpha\) antagonist), showed a reduction in OPG expression after 1 year, but RANKL and the OPG/RANKL ratio remained unchanged.\(^43\) The decreased expression of systemic, but not synovial, OPG that we observed after B cell depletion may be related to effects on a variety of cells. This soluble receptor is produced by for instance bone marrow stromal cells, dendritic cells, endothelial cells, fibroblasts, monocytes, B cells and T cells under the influence of proinflammatory cytokines.\(^37\) The reduction of sRANKL in our patients led to an elevated OPG/sRANKL ratio, as seen in several of the aforementioned clinical studies. The relative expression levels of OPG and RANKL are critical for the regulation of osteoclastic activity and bone resorption and this ratio seems to be the most important predictor of later joint destruction in chronic, destructive arthritis.\(^45\)

We found markedly increased osteocalcin levels 16 weeks after treatment at a borderline level of statistical significance, suggesting an increase in bone formation activity. Consistent with our results, previous studies have shown that serum levels of osteocalcin are negatively correlated with disease activity in RA patients.\(^46\)-\(^47\) Of note, one other study in 46 RA patients did not demonstrate a change in osteocalcin 6 months after rituximab treatment.\(^48\); the reason for this discrepancy is at present unclear. The fact that serum NTx did not change can perhaps be explained by the long disease duration of our cohort, as previous studies have shown that CTx (carboxy terminal telopeptide; a comparable bone resorption marker) levels are decreased in RA patients with a disease duration of more than 10 years. When baseline levels are already low, it is more difficult to demonstrate a decrease.\(^49\) In addition, we cannot exclude the possibility that changes in NTx levels might have been detectable if this biomarker had been tested in urine, but urine samples were unfortunately not available in our study.

In conclusion, the results presented here support a link between B cells and the RANK/ RANKL/OPG system at the site of inflammation and destruction. Depletion of B cells may ultimately interfere with this mechanism, protecting the joints against progressive destruction.
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