Infliximab inhibits bone resorption by circulating osteoclast precursor cells in patients with rheumatoid arthritis and ankylosing spondylitis

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ABSTRACT

Objective: To examine the effects of infliximab on bone resorption by osteoclast precursor cells (OCPs) in patients with rheumatoid arthritis (RA) and ankylosing spondylitis (AS) and to compare the results with changes in disease activity.

Methods: Before and during 24 weeks of infliximab treatment, peripheral blood mononuclear cells of 9 RA and 10 AS patients were seeded onto ivory wafers and adherent cells, including OCPs, were grown in medium promoting osteoclast differentiation. Bone resorption was evaluated morphometrically and correlated to disease activity. A total of 19 healthy individuals were studied in parallel. In addition, biochemical bone markers were assessed in all patients at baseline and after 24 weeks.

Results: OCPs from RA patients showed a higher bone resorption at baseline when compared to AS patients. Blocking of tumour necrosis factor (TNF)α with infliximab resulted in a strong reduction of bone resorption by OCPs in both cohorts and occured faster in RA compared to AS patients. This inhibition coincided with a reduction of clinical disease activity in both patient cohorts and with an increase of serum osteocalcin levels and a relative decrease of collagen crosslinks in RA compared to AS patients.

Conclusion: These results provide an explanation on the cellular level for the anticatabolic effect of TNF neutralisation on bone. The variation in the kinetics of bone resorption by the OCPs in patients with RA and AS suggests disease-specific differences in the type or in the preactivation of OCPs.

Evidence from animal models of chronic arthritis indicates that local and systemic bone loss are the result of enhanced osteoclast-mediated bone resorption, which may be caused by enhanced levels of tumour necrosis factor alpha (TNFα).[1] TNFα promotes the expansion and differentiation of osteoclast precursor cells in TNFα-transgenic mice[2] and stimulates osteoclast differentiation from progenitor cells in synergy with the receptor activator nuclear factor (NF)-κB ligand (RANKL), an obligate growth factor for osteoclast lineage cells.[3–6] By contrast, anti-TNFα treatment was shown to reduce the osteoclast precursor cell (OCP) frequency in psoriatic arthritis[7] and to suppress the expression of RANK in rheumatoid arthritis (RA) peripheral blood mononuclear cells (PBMCs) and of RANKL in RA synovial fibroblasts along with an increase of osteoprotegerin (OPG).[8] As a result, structural joint damage, in psoriatic arthritis[9] as well as in RA patients[10] was reduced.

In the present study, we prospectively studied the effect of infliximab on the resorptive capacity of circulating osteoclast precursor cells, correlated the data with the clinical response and with changes of markers of bone turnover, and compared the effects of the treatment in two diseases, namely in RA primarily affecting the juxtaarticular bone of peripheral articulations and in ankylosing spondylitis (AS) primarily affecting the axial skeleton.

METHODS

Patients and study design

The study included 9 consecutive female patients with RA (mean age 59.2 years (range 45–76), 2 premenopausal and 7 postmeopausal; mean disease duration 9.1 years (range 1–26)) and 10 patients with AS (mean age 34 years (range 30–51), 2 premenopausal females and 8 males; mean disease duration 5.4 years (range 1–10)), who were eligible for anti-TNFα treatment with infliximab, due to active disease. Infliximab was administered intravenously at baseline and after 2, 4, 6, 12 and 24 weeks (3 mg/kg in RA patients and 5 mg/kg in AS patients). A total of 19 untreated gender-and age-matched healthy individuals served as controls. Clinical disease activity was determined using the Disease Activity Score based on 28 joints (DAS28) for RA patients,[11] and the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) for AS patients[12] before and at 2 days as well as 2, 12 and 24 weeks after the onset of treatment. RA and AS patients who had received baseline medication prior to the start of infliximab treatment, continued their concomitant medication throughout the study period. Within the RA cohort, six patients received <7.5 mg prednisone daily, five patients were treated with non-steroidal anti-inflammatory drugs (NSAIDs), eight were treated with methotrexate (MTX) and two patients obtained sulfasalazine. Within the AS patient cohort, seven patients received NSAIDs and four were treated with MTX because of recurrent peripheral synovitis. Calcium and vitamin D3 supplements were provided to six out of nine RA patients. Concomitant medications such as corticosteroids >7.5 mg prednisone equivalent daily, bisphosphonates, anti-epileptics, heparin, oestrogens, testosterone, thiazids and angiotensine converting enzyme inhibitors were prohibited throughout the study duration. The diagnosis of osteoporosis, either documented by DEXA (dual energy *x* ray absorptiometry) and/or based on clinical manifestations such as fractures, as well as major systemic illnesses were further exclusion criteria for patients and healthy controls. The

postmenopausal status of women was well balanced among patients and controls. Blood was drawn from each individual before and at the time points mentioned above for routine laboratory tests (erythrocyte sedimentation rate, C-reactive protein, serum calcium, phosphate, albumin, creatinin, alkaline phosphatase, osteocalcin) and for experimental biochemical markers of bone turnover (RANKL, OPG). Deoxypyridinolline (DPD) crosslinks were measured routinely in urine before and after infliximab treatment. Serum, plasma and urine samples were stored at −70°C for blinded collective analysis at the end of the study. This study was approved by the local ethics committee of the canton of Bern and a written informed consent was obtained from all patients and control individuals.

Resorption assay for osteoclast precursor cells

To assess the bone resorptive potential of OCPs from peripheral blood, a resorption assay as described in previously published protocols[13 14] was used. Accordingly, PBMCs were isolated from 16 ml EDTA-blood by Ficoll-Hypaque density gradient centrifugation, washed twice with phosphate-buffered saline (PBS) and diluted in alpha minimal essential medium (alpha-MEM; GIBCO BRL, Life Technologies, Basel, Switzerland) containing 1% penicillin/streptomycin and 10% foetal bovine serum (FBS; INOTECH AG, Dottikon, Switzerland). PBMCs (2.5×10^5/0.5 ml) were seeded onto 15 ivory wafers distributed in 48-well plates (Falcon, Fisher Scientific AG, Wohlen, Switzerland). After adherence of mononuclear cells for 2 h at 37°C in an atmosphere of 5% CO_2, culture medium (alpha-MEM, 10% FBS, 1,25-dihydroxycholecalciferole 10^{-8} M from Roche (Basel, Switzerland); dexamethasone 10^{-8} M, colony-stimulating factor-1 (CSF-1; 25 ng/ml from Chiron Corporation, Emeryville, California, USA) was added. The medium was changed 3 times a week. Cells were incubated for 1 week with culture medium and for another 2 weeks with culture medium supplemented with human RANKL (50 ng/ml; Pepro Tech EC LTD, London, UK). With each cell preparation, three wafers were incubated without RANKL to serve as negative controls. After 3 weeks, the cultures were stopped and the ivory wafers were stained with 0.5% toluidine blue. Thereafter, the 15 experimental and the 3 control wafers were glued onto glass slides for microscopic analysis. The slides were blinded and the resorption areas on the ivory wafers were determined morphometrically, using a grid with 480 intersection points on each wafer, by an external person. Adherent PBMCs from untreated gender- and age-matched healthy individuals were run in parallel. Baseline pit count ratios patient/healthy control (p/c) before starting infliximab treatment were set as 1. Then, the pit count ratios p/c at each time point upon infliximab treatment were related to the corresponding baseline ratio.

Measurement of markers of bone metabolism

Serum levels of RANKL and OPG in RA and AS patients were determined by ELISA (Biomedica, Vienna, Austria; lower detection limit 0.08 pmol/litre and 0.14 pmol/litre respectively). Osteocalcin serum levels (Roche Diagnostics, Rotkreuz, Switzerland) and urinary secretion of DPD (Diagnostics Products Corporation, Los Angeles, California, USA) were measured by a chemiluminescence immunoassay. Serum levels of calcium, anorganic phosphate and alkaline phosphatase were determined by standard analytical methods.

Statistical analysis

A statistical analysis was performed using the Wilcoxon ranked sum test and differences reached significance at levels of $p<0.05$.

RESULTS

Patient characteristics prior to infliximab treatment

Table 1 shows the baseline characteristics of the patients included in the present study. As anticipated, the majority of RA patients were postmenopausal middle-aged females, whereas the AS group mainly included younger males. Patient cohorts differed significantly in age ($p<0.001$), the resorptive potential of PBMCs ($p<0.02$), serum osteocalcin levels ($p<0.05$), urinary secretion of DPD crosslinks ($p<0.005$) and concomitant low dose steroid medication. There was no significant difference between the groups, with regard to disease duration, acute phase reaction and bone mineral density. As documented by the moderate elevation of the DAS28 and BASDAI, RA and AS patients exhibited an active disease at the time of anti-TNFα treatment initiation.

Bone resorption potential of PBMCs with infliximab treatment

Blockade of TNFα resulted in a reduction of bone resorption by PBMCs in both patient cohorts, although the kinetics of the inhibitory effect of infliximab infusions on OCP activity in RA patients was different from that seen in AS patients when compared to the baseline values. For each patient and time point, the total bone resorption in the cultures of PBMC was normalised against the bone resorption of PBMC from a gender and age-matched healthy control individual. The inhibition of osteoclast resorptive activity was more pronounced at 2 and 14 days after the first infliximab infusion in RA patients (fig 1;

Table 1 Patients baseline characteristics

	RA (n = 9)	AS (n = 10)	Significance
Sex:			
Female (n)	9	2	
Male (n)	0	8	
Ethnic group:			
Caucasian (n)	8	7	
Arab (n)	1	2	
Indian (n)	0	1	
Hormonal status:			
Premenopausal	2	2	
Postmenopausal	7	0	
Prednisone ⩽7.5 mg/day	6	0	
Age, years*	59.2 (11.4)	38.8 (7.6)	p<0.001
Disease duration, years	9.1 (7.8)	6.2 (5.4)	NS
Clinical assessment:			
DAS28 (SD) (range)	4.60 (1.02) (2.89 to 5.19)	–	
BASDAI (range)	–	5.57 (1.61) (3.25 to 7.48)	
Laboratory tests:			
ESR, mm/h	16.1 (4.3)	28.4 (8.2)	NS
CRP, mg/litre	15.7 (4.5)	17.2 (5.4)	NS
ANA+ (%)	22.2	0	
RF+ (%)	77.8	0	
HLA-B27+ (%)	0	100	
Osteocalcin, ng/ml	12.4 (3.5)	20.8 (12.5)	p<0.05
DPD/creatinine, nmol/mmol	9.9 (4.9)	6.2 (1.7)	p<0.005
OCP activity, pit counts t/c†	7.8 (3.8)	2.1 (0.9)	p<0.02

All values are mean (SD) unless otherwise stated.
†t/c = treated over control.
ANA, antinuclear antibody; AS, ankylosing spondylitis; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; CRP, C-reactive protein; DAS28, 28-joint Disease Activity Score; DPD, deoxypyridinolline; ESR, erythrocyte sedimentation rate; HLA, Human leukocyte antigen; NS, not significant; OCP, osteoclast precursor cells; RA, rheumatoid arthritis; RF, rheumatoid factor.

82% and 84%, p<0.001 for intragroup differences from baseline) compared to AS patients (fig 1; 38% and 70%, p<0.001 and p<0.001 for intragroup differences from baseline). The intergroup differences in the reduction, however, were statistically significant only 2 days after the first infliximab infusion (p<0.05). After 3 and 6 months of treatment, the reduction of the osteoclastogenic potential of the OCPs was similar in both patient cohorts (90% in RA vs 72% in AS and 90% in RA and 88% in AS respectively; p<0.001 for intragroup differences from baseline).

Disease activity upon infliximab treatment

Figure 2 shows the reduction of disease activity in patients treated with infliximab, which was achieved more rapidly (p<0.001 at day 2 for intragroup comparison) and proved more significant over time in AS (p<0.001 at week 2, 12 and 24 for intragroup comparisons) as compared to RA patients (not significant at day 2; p<0.01 to p<0.001 from week 2 to week 24 for intragroup comparisons).

Changes in biochemical markers of bone turnover with infliximab treatment

Before starting infliximab treatment and 24 weeks thereafter, serum levels of calcium, anorganic phosphate, alkaline phosphatase, osteocalcin, RANKL, OPG and urine levels of deoxypyridinolline were measured. Table 2 shows the changes in markers of bone metabolism during the study period. We observed a significant increase of serum osteocalcin levels (mean (SD) 12.4 (3.5) vs 18.2 (6.4) ng/ml; p<0.05 for intragroup comparison) and a relative decrease of the urinary secretion of DPD crosslinks in RA compared to AS patients after 6 months of infliximab treatment (RA: 9.9 (4.9) vs 8.2 (4.0) nmol DPD/mmol creatinine; AS: 6.2 (1.7) vs 5.7 (2.9) nmol DPD/mmol creatinine; p<0.035 for intergroup comparison after 6 months and no significant difference for intragroup comparisons compared to baseline), whereas in AS patients there was no change in these bone turnover markers. There were no significant changes of serum levels of calcium, anorganic phosphate, alkaline phosphatase, RANKL and OPG.

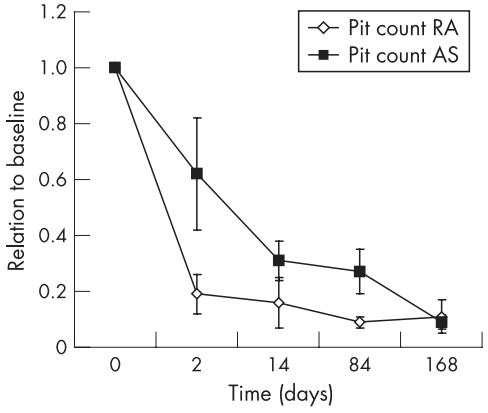

Figure 1 Resorptive activity of blood osteoclast precursor cells (OCPs) and disease activity in rheumatoid arthritis (RA) and ankylosing spondylitis (AS) patients before and upon infliximab treatment. Peripheral blood mononuclear cell (PBMC) OCPs from 9 RA and 10 AS patients were seeded onto ivory wafers, adherent cells were incubated with conditioned medium including 1,25-dihydroxy-cholecalciferol 10^{-8} M, dexamethasone 10^{-8} M, CSF-1 25 ng/ml for 1 week and thereafter with receptor activator nuclear factor (NF)-κB ligand (RANKL) (50 ng/ml) for another 2 weeks. After 3 weeks cell cultures were stopped and ivory wafers were stained with 0.5 % toluidine blue. Resorption areas on the ivory wafers were counted morphometrically under the microscope and the number of pit counts of RA and AS OCPs was adjusted to pit counts of their corresponding healthy control counterparts. In relation to baseline p/c ratios results are given as means of ratios of patient pit counts and healthy control pit counts (p/c) after 2, 14, 84 and 168 days of infliximab treatment and baseline (standard error of the mean) (ratio before treatment was set at 1; RA: p<0.001 for intragroup comparison at each time; AS: p<0.001 after 2 days, p<0.001 after 14 days, p<0.001 after 84 and 168 days for intragroup comparison). The intergroup differences in the reduction of the OCP pool size between RA and AS patients were only statistically significant 2 days after the first infliximab infusion (p<0.05).

DISCUSSION

The first finding of this study is the demonstration of a larger bone resorptive potential of OCPs in peripheral blood from RA patients with active disease compared to AS patients with active disease. Several factors may contribute to this difference: postmenopausal status of the majority of RA patients, concomitant glucocorticoid medication and longstanding inflammatory disease. By contrast, apart from two young premenopausal women, the AS patient group mainly consisted of males. Glucocorticoids inhibit intestinal calcium absorption and bone formation[15] and evidence suggests that glucocorticoids and 1,25 dihydroxyvitamin D3 may cause an increase in the number of OCPs.[16] OCPs with the $CD11b^{+}/Gr\text{-}1^{-/lo}$ phenotype are common precursors for macrophages, dendritic cells, and osteoclasts and their number may increase non-specifically in chronic inflammatory diseases. This was previously confirmed by the increase in the number of circulating OCPs ($CD11b^{+}/CD14^{+}$) in psoriatic arthritis patients.[7] Furthermore, the number of circulating OCPs was elevated in TNF-Tg mice with established arthritis.[2]

Administration of TNFα to wild-type mice increases the proliferation and differentiation of OCPs in the bone marrow by inducing an up-regulation of c-Fms expression and TNFα promotes the release of OCPs from the bone marrow, which is associated with decreased SDF-1 production by marrow stromal cells.[5]

The second finding of this study is the rapid reduction of the bone resorptive potential of circulating OCPs in response to TNF blockade that is accompanied by a clinical improvement of inflammatory disease activity in RA and in AS. Based on the results of the in vitro bone resorption assay it can be assumed that the number of circulating OCPs is reduced by the anti-TNFα treatment. After the culture of the cells for 2 weeks with CSF-1 and RANKL a maximal stimulation of osteoclastic bone resorption is achieved, making the final extent of resorption dependent on the number of original OCPs only.

The different kinetics of the reduction of the bone resorptive capacity of OCPs upon anti-TNFα treatment in RA and AS patients may suggest that target cell subpopulations of OCPs might have been affected differently. It will be interesting to analyse whether a disease-specific inhibition of OCP recruitment from the bone marrow has occurred or whether enhanced apoptosis or a different susceptibility of OCPs to RANKL based on differential regulation of RANK expression on OCPs upon anti-TNFα treatment may have played a role. It is also possible that concomitant glucocorticoid stimulation of RA OCPs played a role and did render these cells more susceptible to TNFα blockade. By contrast, AS patients were not treated with glucocorticoids and started with a significantly lower OCP bone resorptive capacity, which was less rapidly reduced by TNF

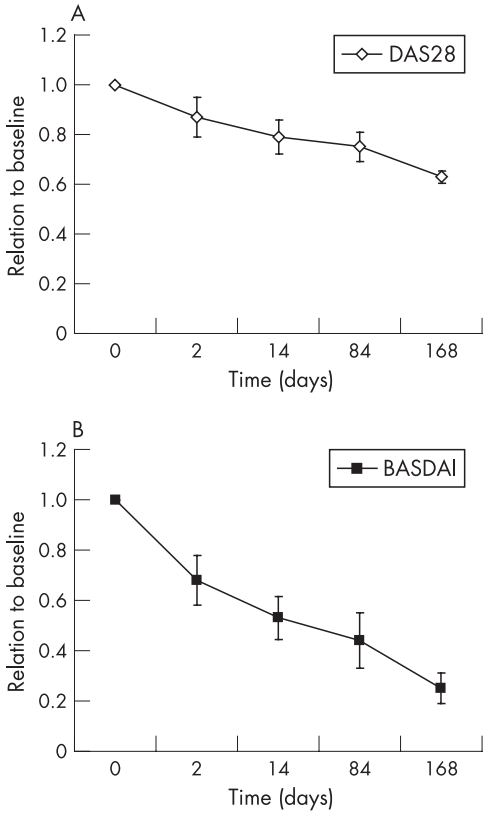

Figure 2 Disease activity in rheumatoid arthritis (RA) and ankylosing spondylitis (AS) patients before and upon infliximab treatment. Disease activity in RA and AS patients was assessed by 28-joint Disease Activity Score (DAS28) (A) and Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) (B) respectively. Results are given as mean of ratio values at corresponding time points and at baseline (standard error of the mean) (ratio before treatment was set as 1; RA: not significant after 2 days, $p<0.01$ after 14 days and $p<0.001$ after 84 and 168 days; AS: $p<0.001$ after 2 days, $p<0.001$ to $p<0.001$ from day 14 to 168 for intragroup comparisons).

blockade but can be equated with that seen in RA patients on maintenance treatment.

The increase in the synovial expression of RANKL in active states of RA, which is paralleled by a decrease in OPG levels, and the trend to a decrease in RANKL expression in inactive states of RA[16] suggests a local regulation of the differentiation of OCPs into osteoclasts by RANKL. This local regulation of RANKL and OPG is in agreement with our finding that no significant changes in circulating levels of RANKL and OPG in RA and AS patients upon anti-TNFα treatment were detected. TNFα and RANKL act synergistically on osteoclast development, with only permissive levels of RANKL being required for TNFα dependent cell differentiation. Therefore, the observed reduction of the bone resorptive capacity of OCPs upon anti-TNFα treatment may be caused by the blockade of the RANKL-independent stimulatory effect of TNFα on the number of circulating OCPs.[17] A second possibility is the decrease in the expression of RANKL by bone marrow stromal cells due to the removal of TNFα, the expression of RANKL by stromal cells depending on the synergistic action of TNFα and interleukin (IL)-1.[18]

The increase in the number of $CD11b^+/CD14^+$ cells in peripheral blood is indicative for the inflammatory status of a patient and may determine the number of osteoclasts formed in response to locally released osteoclastogenic cytokines. The divergent ratios of baseline levels of serum osteocalcin and urine DPD crosslinks between both patient cohorts support the finding discussed above, namely that prior to TNF blockade, RA patients exhibited a greater bone resorptive/catabolic activity than did AS patients. This increase in metabolism might put RA patients at a higher risk of bone loss and may contribute to their rapid response, as seen by the reduction of OCP bone resorptive activity. The significant increase of serum osteocalcin in RA patients upon treatment with anti-TNFα may reflect the release of an initial inhibitory effect of TNFα on osteoblast metabolic function.[19]

Collectively, this is the first study demonstrating: (1) that PBMCs from RA patients with active disease exhibit a stronger bone resorptive capacity of circulating OCPs when compared to cells from AS patients with active disease, and (2) that TNFα blockade with infliximab markedly reduces the bone resorptive potential of circulating OCPs along with the suppression of inflammatory disease activity in RA and AS patients. Therein, a causal interrelationship through the interaction of circulating TNFα and/or chemokines with bone marrow stromal cells is very likely. There remains a striking finding in RA patients, namely the rapid and sustained loss of circulating OCPs that can differentiate into osteoclasts and seem to depend entirely on TNFα. Therefore, it is planned to further characterise RA OCPs phenotypically as well as functionally and to examine whether this subpopulation is particularly sensitive to (i) anti-TNFα treatment with respect to a loss of appropriate recruiting signals from the bone marrow, (ii) early central as well as peripheral apoptosis, or (iii) a loss of the permissive effect of RANKL for differentiation into mature osteoclasts.

Table 2 Changes in biochemical markers of bone turnover upon infliximab treatment

	RA (n = 9)		AS (n = 10)		Change difference	
Marker	**Baseline**	**6 months**	**Baseline**	**6 months**	**Intragroup**	**Intergroup**
Calcium (mmol/litre)	2.38 (0.10)	2.33 (0.09)	2.53 (0.17)	2.38 (0.12)	NS	NS
Anorganic phosphate (mmol/litre)	1.08 (0.17)	1.07 (0.11)	1.05 (0.12)	1.14 (0.15)	NS	NS
Alkaline phosphatase (U/litre)	72.4 (19.6)	67.0 (20.0)	91.0 (26.8)	73.0 (20.0)	NS	NS
RANKL (pmol/litre)	0.21 (0.04)	0.22 (0.04)	0.23 (0.07)	0.21 (0.07)	NS	NS
OPG (pmol/litre)	1.10 (0.60)	0.92 (0.37)	0.96 (0.63)	0.94 (0.54)	NS	NS
Osteocalcin (ng/ml)	12.4 (3.5)	18.2 (6.4)	20.8 (12.5)	22.6 (10.1)	$p<0.05$ RA	NS
DPD (mmol/mmol creatinine)	9.9 (4.9)	8.2 (4.0)	6.2 (1.7)	5.7 (2.9)	NS	$p<0.035$

Values are given as mean (SD) of given number of patients.
AS, ankylosing spondylitis; DPD, deoxypyridinolline; OPG, osteoprotegerin; NS, not significant; RA, rheumatoid arthritis; RANKL, receptor activator nuclear factor (NF)-κB ligand.

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