The Tumor Necrosis Factor-α-blocking Agent Infliximab Inhibits Interleukin 1ß (IL-1ß) and IL-6 Gene Expression in Human Osteoblastic Cells

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ABSTRACT. *Objective.* Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine involved in the pathogenesis of several rheumatic diseases, including rheumatoid arthritis (RA), associated with systemic bone loss and subchondral bone erosions. TNF-α-blocking agents such as infliximab have been successful in treatment of disease-modifying antirheumatic drug-resistant rheumatic diseases. Infliximab therapy in RA also had beneficial effects on local bone destruction and bone mineral density. We assessed effects of infliximab treatment on the bone tissue compartment and cytokine profile expression *in vitro*.

Methods. Osteoblast-like cells were exposed for 24 h to sera of RA patients collected at baseline and after 1 month (T1) and 3 years (T2) of infliximab treatment. Total RNA was extracted, and expression of interleukin 1ß (IL-1ß), IL-6, and osteoprotegerin (OPG) was measured by RT-PCR.

Results. IL-1ß gene expression was significantly reduced by the T1 serum, and the same decrease was elicited by the T2 serum. IL-6 downregulation was evident with the T2 serum. OPG was unaffected.

Conclusion. The finding of downregulation of inflammatory cytokines was interesting, particularly IL-6, which plays a crucial role in arthritis-related bone loss due to its involvement in osteoclast recruitment and activation. These results may represent a biological explanation and a link for the clinical observation of the beneficial effects of anti-TNF-α agents on the progression of rheumatic diseases at the bone level. (J Rheumatol First Release July 1 2009; doi:10.3899/jrheum.081321)

Key Indexing Terms:
ANTI-TUMOR NECROSIS FACTOR-α   INFLIXIMAB   OSTEOBLASTS
INTERLEUKIN 1ß   INTERLEUKIN 6   OSTEOPROTEGERIN

Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine that plays a crucial role in the pathogenesis of several rheumatic diseases including rheumatoid arthritis (RA)[1-4]. RA is a chronic inflammatory disease of uncertain cause characterized by synovial hyperplasia, inflammation, and joint destruction[5]. The excessive growth of rheumatoid synovial fibroblasts is thought to be responsible for the formation of pannus, which invades adjacent tissues including cartilage and bone. Moreover, a common finding in chronic inflammatory diseases is systemic bone loss that is aug-mented by the low level of physical activity as well as by the pharmacological treatments used, generally recognized to inhibit osteoblast activity *in vitro* and *in vivo*[6]. The bone erosion characterizing RA is related to the bone-acting inflammatory cytokines of the joint microenvironment: cytokines such as interleukin 1ß (IL-1ß) and IL-6 produced by osteoblasts in response to proinflammatory stimuli such as TNF-α are responsible in RA for chronic inflammation, osteoclastogenesis, and bone resorption[7-9]. Moreover IL-1ß, IL-6, and IL-23 (a member of the IL-6 family) promote differentiation of Th17[10], thus influencing the Th17 pathway that is prominently involved in inflammation and autoimmunity, and the consequent Th17/Treg balance that has recently been postulated to play a pivotal role in the destructive processes that characterize RA[11].

Biological agents are large proteins manufactured to target a specific protein, antibody, or receptor. Among these, TNF-α-blocking agents have recently been developed and successfully used in the treatment of rheumatic diseases resistant to disease modifying antirheumatic drugs (DMARD), improving patients' quality of life[12-14]. It has been shown that they slow the progression of RA and provide relief from pain and inflammation, reducing the socioe-

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conomic impact of such diseases related to patients' lower work attendance.

It has been reported that anti-TNF-α treatment in patients with RA and ankylosing spondylitis reduces osteoclastogenesis[15] and has beneficial effects on local bone destruction as well as on femoral and lumbar bone mineral density, and markers of bone turnover[16,17].

One of the most-used TNF-α-blocking agents is infliximab, a chimeric (75% mouse/25% human) anti-TNF-α monoclonal antibody composed of the constant region of human immunoglobulin and 2 murine variable regions targeted to TNF-α. It comprises 2 binding sites for TNF-α and may activate complement-mediated cell lysis[12].

To assess the influence of longterm anti-TNF-α treatment on the bone tissue compartment and the cytokine profile induced in osteoblastic cells, we set up an *ex vivo* model of osteoblast-like cells challenged with sera derived from RA patients at baseline and during the course of treatment with infliximab.

MATERIALS AND METHODS

Patients. Sera were obtained from peripheral blood collected by venipuncture from 10 subjects (8 women, 2 men) with their informed consent. The patients were diagnosed with erosive RA according to the American College of Rheumatology criteria[18], and had been treated with infliximab 3 mg/kg body weight, intravenous infusion, every 2 weeks for the first month and once a month subsequently. All had been treated previously with at least 3 different DMARD with no improvement in their clinical condition.

Blood was obtained at baseline (i.e., before the beginning of treatment), after 1 month (T1), and after 3 years (T2) of infliximab treatment. All subjects continued the same treatment established before they underwent anti-TNF therapy. In particular, at the onset of infliximab therapy, all patients were under medication with methotrexate from at least 3 months. All observed a washout period for nonsteroidal antiinflammatory drugs of at least 2 weeks. Subjects taking medications that can interfere with bone metabolism (such as bisphosphonates, sex hormones, parathyroid hormone) were not included. Finally, occasional therapy with glucocorticoids was limited to < 5 mg/day throughout the study.

Materials. Tissue culture plates and flasks and all other disposable materials were from Corning, UK. Tissue culture media RPMI-1640 and all other chemicals were from Sigma-Aldrich, Italy. GeneAmp RNA polymerase chain reaction (PCR) kit components were purchased from Applied BioSystems (Monza, Italy). The specific primers used for amplification were obtained from Primm (Milan, Italy).

Cell culture and treatment. The human osteoblast-like cell line MG-63 (CRL 1427; American Type Culture Collection, USA) was employed[19,20]. Cell cultures were maintained in a humidified 5% CO_2 atmosphere at 37°C in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (HI-FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell viability was evaluated by Trypan blue exclusion test.

Cells were plated in 6-well plates at 8×10^4 cells/well and challenged with different concentrations (5%, 10%, 20%) of patient sera for 24 or 48 hours in 0.5% HI-FCS medium. Controls were represented by patient serum at baseline.

Gene expression. Gene expression was measured using the semiquantitative RT-PCR approach[21]. At the end of the various treatments, total RNA was extracted using TRI reagent. RNA quality was evaluated using a Bioanalyzer 2100 (Agilent Technologies, Milan, Italy) and samples with RNA integrity number > 9 were selected for use[22]. Total RNA (1 μg) was used as the template for cDNA synthesis. cDNA amplification was carried out with specific primers: IL-1ß forward primer, AAA CAG ATG AAG TGC TCC TTC AGG; reverse, TGG AGA ACA CCA ATT GTT GCT CCA (391 bp); IL-6 forward, ATG AAC TCC TTC TCC ACA AGC GC; reverse, GAA GAG CCC TCA GGC TGG ACG G (628 bp); osteoprotegerin (OPG) forward, GGG GAC CAC AAT GAA CAA GTT G; reverse, AGC TTG CAC CAC TCC AAA TCC (408 bp); and 18S forward, GAA GGT CGG AGT CAA CGG ATT TG; reverse, CAT GTG GGC CAT GAG GTC CAC CAC (149 bp). Amplification products were analyzed after polyacrylamide gel electrophoresis and silver staining. Quantification was performed by densitometric analysis (US National Institutes of Health Image Analyzer software).

Statistical analysis. Data represent mean ± SD of 3 different experiments performed in duplicate. Results are expressed as the ratio between the level of target gene expression following different treatments and the level of expression obtained in controls. Statistical analysis was carried out by ANOVA with a between-within design.

RESULTS

Patients' characteristics and disease features are outlined in Table 1. As different medications had been used before therapy with the biological drug was established, the average timespan from disease onset to the beginning of treatment was 9.6 ± 5.6 years. Biochemical markers of inflammation were already decreased after the first month of therapy. At the 3-year followup the laboratory measures had diminished by 60%–90%, while clinical indexes indicated partial or complete remission of symptoms.

Cell culture and treatment. On the basis of previous time- and dose-dependent experiments (data not shown) using serum at 5%, 10%, and 20% for 24 and 48 hours, the concentration used in the subsequent experiments was set at 10% and the length of stimulation at 24 hours.

Figure 1 shows the effects of a 24-hour challenge of MG-63 with serum from patients with RA and treated with infliximab. IL-1ß gene expression was already significantly diminished by exposure to serum obtained after 1 month of treatment (p < 0.01 vs baseline). This decrease persisted over time, and it was similar in the serum obtained after 3 years of treatment. IL-6 was slightly downregulated by 1-month treatment serum, but it decreased to 70% after 3 years (p < 0.01 vs baseline). OPG expression was also diminished after 1 month and after 3 years, but the extent of the decrease was minor and did not reach statistical significance.

DISCUSSION

The aim of our study was to verify the effect of treatment with the anti-TNF-α drug infliximab at the osteoblast level *ex vivo*, with an experimental model reproducing the local setting in the course of RA. Osteoblast-like cells were challenged with sera from patients at baseline and at 2 timepoints during the therapy with infliximab. We observed a decreased expression of bone-related cytokines. The inhibition was already evident after 1 month of treatment, suggesting that the agent has an early effect on the circulating factors that can stimulate activation of bone turnover. The

Variable	T0	T1, Δ		T2, Δ	
M/F ratio	0.25				
Age at disease onset, yrs	33.6 ± 11.3				
Age at infliximab therapy onset, yrs	44.6 ± 9.8				
Laboratory values					
ESR, mm/h	38.8 ± 25.1	11.0 ± 6.4	–71.6	12.8 ± 7.3	–67.0
CRP, mg/l	49.5 ± 34.1	5.6 ± 5.4	–88.6	3.1 ± 1.5	–93.7
WBC, cell/mm³	11522 ± 2514	8478 ± 1146	–26.4	7460 ± 2203	–35.3
Clinic values					
Disease Activity Score	5.9 ± 1.1	3.0 ± 0.4	–49.5	1.4 ± 0.4	–77.1
Swollen joints, n	10.2 ± 4.7	5.2 ± 2.5	–49.0	1.0 ± 1.4	–90.2
Painful joints, n	16.0 ± 5.5	11.0 ± 4.1	–31.3	2.8 ± 2.7	–82.5

T0: before therapy (baseline); T1: after 1 mo of therapy; T2: endpoint of *in vitro* experiment (3 yrs). Δ = % difference vs T0; $p < 0.001$. ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; WBC: white blood cell count.
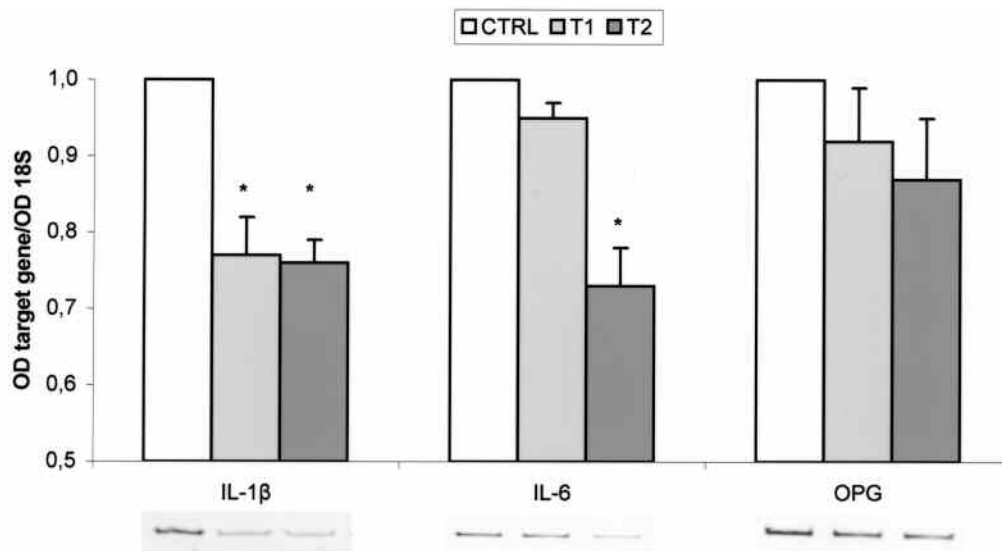

Figure 1. IL-1ß, IL-6, and osteoprotegerin (OPG) gene expression measured by RT-PCR in MG-63 cells challenged for 24 h with 10% serum from patients with RA treated with infliximab. mRNA levels are reported as target gene/18S ratio and normalized to control (CTRL). Data represent mean ± SD of 3 experiments performed in duplicate; one representative result of 3 experiments is shown below the corresponding data group. *$p < 0.01$ versus control (baseline condition). T1: 1 month; T3: 3 years.

experimental data were paralleled by the decrease of both laboratory markers and disease indexes at the same timepoints. It is known from clinical observations that monoclonal antibodies generally show a greater efficacy at the onset of therapy[12]. TNF-α induces IL-1ß production, which contributes to disease development in RA. The inhibition of IL-1ß expression in osteoblastic cells is relevant with respect to the events occurring in the bone compartment[23,24]. Moreover, it is well known that in response to IL-1ß and TNF-α, osteoblasts produce the cytokine IL-6, which in turn recruits and activates osteoclasts leading to bone resorption[25,26].

The role of IL-1ß in the pathophysiology of RA is crucial because its activity is exerted on different cell types present in the joint spaces. Articular cartilage is the main target of IL-1ß-induced expression of inflammation mediators and metalloproteases[27,28]. A study by Zwerina, *et al*[29] in an experimental model of erosive arthritis represented by transgenic mice heterozygous for TNF-α demonstrated that monotherapy with infliximab or anakinra (IL-1Ra) was unable to completely arrest synovial inflammation, while instead the anti TNF-α/IL-1Ra combination was effective. We observed a 25% decrease in IL-1ß expression in osteoblasts after 1 month of therapy with infliximab. It can be assumed that at the articular level the inhibition of circulating TNF-α not only reduces IL-1ß production, but also

decreases the 2 cytokines' synergistic effects. The downregulation of IL-6 during exposure to infliximab-treated patient sera is also a relevant finding, as this cytokine, besides being an indicator of local inflammation, is a major stimulator of osteoclast activity[25]. Its decrease once IL-1ß downregulation is established may therefore represent another beneficial effect of anti-TNF-α therapy in RA and in particular on bone erosion. The reduction in IL-6 expression was detectable only after a longer treatment period, because the system needs to be "turned off." This finding gains relevance because the clinical indexes and also the patients' radiological erosion scores were significantly reduced during the course of therapy (data not shown).

OPG and the receptor activator of nuclear factor-κB ligand (RANKL) are inflammatory cytokines traditionally linked to regulation of bone remodeling. The OPG/RANK/RANKL axis is essential for osteoclast activation that drives bone loss in inflammatory arthritis[30,31]. However, it is not possible to speculate on an explanation for the minor effect on OPG expression in our experimental model, as it was not significantly altered by the treatment. The OPG/RANK/RANKL system is complex and its regulation is orchestrated by a variety of factors[32,33].

The downregulation of inflammatory cytokine expression we observed is relevant in particular with respect to IL-6, which plays a crucial role in arthritis-related bone loss. Despite the limitations of our experimental model, these findings may represent a biological explanation and a link for the clinical observation of the beneficial effects of anti-TNF-α agents on the progression of rheumatic diseases at the bone level.

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