Osteoprotegerin and Receptor Activator of Nuclear Factor-κB Ligand mRNA Expression in Patients with Rheumatoid Arthritis and Healthy Controls

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ABSTRACT. ***Objective***. To further understand the role of osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANK-L) in rheumatoid arthritis (RA), we studied the levels of RANK-L and OPG mRNA in peripheral blood mononuclear cells (PBMC) and synovial tissue of patients with RA and controls.

Methods. RANK-L and OPG mRNA levels were measured in PBMC and CD4+/CD8+ T cell subsets of patients with chronic RA, osteoarthritis (OA), and healthy controls, using quantitative real-time polymerase chain reaction. OPG and RANK-L mRNA levels were measured in paired blood and synovial tissue samples of patients with early, untreated RA at 2 timepoints with an interval of 16 weeks.

Results. RANK-L mRNA levels were significantly higher in PBMC of patients with early and chronic RA compared to healthy controls. Contrary to healthy controls, RANK-L mRNA levels in patients with chronic RA were mainly of CD4+ T cell origin. OPG mRNA was observed in the blood of all (17/17) early RA patients, but could not be detected in chronic RA patients (0/14) or in patients with OA (0/8). Three out of 17 healthy controls showed measurable levels of OPG mRNA. The OPG/RANK-L ratio tended to be higher in the synovium than in the PBMC of early RA patients. RANK-L mRNA in synovial tissue was mainly of non-T cell origin.

Conclusion. Since RANK-L and OPG mRNA levels are elevated in PBMC of RA patients, and CD4+ T cells are the major contributors to RANK-L mRNA expression, mononuclear cells in patients with RA may be involved in the pathways that regulate bone metabolism. (J Rheumatol 2004;31:1483–90)

Key Indexing Terms:
RHEUMATOID ARTHRITIS — OSTEOPROTEGERIN
RECEPTOR ACTIVATOR NUCLEAR FACTOR KAPPA B LIGAND — SYNOVIUM

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease, characterized by progressive destruction of cartilage and underlying bone in the affected joints. Proteinases secreted by activated synoviocytes and chondrocytes, together with bone resorbing osteoclasts, have been described as key regulators of this destruction[1,2]. Receptor activator of nuclear factor-κB ligand (RANK-L), a member of the tumor necrosis factor (TNF) family, and its receptor, receptor activator of nuclear factor-κB (RANK), are key molecules for bone remodelling and are essential for the development and activation of osteoclasts (as reviewed[3,4]). RANK-L is expressed by osteoblasts, synovial fibroblasts, and activated T cells in both soluble and membrane bound form[5]. Binding of RANK-L to its receptor on osteoclast precursor cells triggers the differentiation of these cells into mature osteoclasts, enhances osteoclast activity, and prevents osteoclast apoptosis, all resulting in an increased osteoclast pool and increased bone degradation[6]. Osteoprotegerin (OPG), like RANK, is a member of the TNF-receptor family[7]. OPG is secreted as a soluble protein by osteoblasts and marrow stromal cells[8,9]. This "decoy" receptor for RANK-L prevents the ligand-receptor interaction that leads to inhibition of osteoclastogenesis, prevention of osteoclast activation, and reduction of osteoclast survival[10]. Thus, the balance between OPG and RANK-L regulates bone resorption[11].

T cells and synoviocytes are considered to trigger osteoclastogenesis in rheumatoid synovium[2,12,13]. Consistent with this, multinucleated giant cells that express specific osteoclast surface markers are formed inside the synovial tissue without contact with the bone and in the absence of osteoblasts[14]. These cells were also observed in primary

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cultures of rheumatoid synovial tissue[15], and in co-cultures of rheumatoid synovial fibroblasts (expressing RANK-L) and peripheral blood mononuclear cells (PBMC)[2]. Further, Weitzmann, *et al* have shown that activated human T cells stimulate osteoclast formation *in vitro*[16]. This suggests that activated T cells present in the synovium may initiate osteoclastogenesis through expression of RANK-L. Further evidence for a role of activated T cells in bone erosion comes from adjuvant arthritis (AA), an animal model of RA, where it has been demonstrated that systemic activation of T cells *in vivo* leads to a RANK-L-dependent increase in osteoclastogenesis that is followed by bone destruction[17]. Interestingly, in both human and rat experiments, all effects of T cells on osteoclasts could be blocked by the administration of OPG. Activated T cells also produce proinflammatory cytokines such as TNF-α, interleukin 1 (IL-1), and IL-11, all of which can induce RANK-L expression in osteoblasts and bone marrow stromal cells[18]. Thus, it appears that T cells play an active role in bone resorption directly through RANK-L expression and indirectly via expression of proinflammatory cytokines that mediate RANK-L expression in non-T cells.

Recent clinical studies with a single-dose administration of OPG to women with postmenopausal osteoporosis showed a profoundly reduced bone turnover for a sustained period, suggesting that OPG may be effective in the treatment of diseases characterized by increased bone resorption, including RA[19,20].

To further understand the role of OPG and RANK-L in RA, we measured OPG and RANK-L mRNA levels in blood of patients with early and chronic RA, osteoarthritis (OA), and healthy controls. Paired blood and synovial tissue samples of early arthritis patients were also analyzed. Our data show a difference in the OPG/RANK-L expression in patients and controls, which suggests that these molecules may play a role in the pathogenesis of RA.

MATERIALS AND METHODS

Study population. Seventeen healthy control subjects (31 ± 2 yrs), 17 early RA patients, 14 chronic RA patients, and 8 OA patients were included in this study (Table 1). Patients were informed about the purpose of the study and gave written consent. Heparinized blood was obtained from all individuals. Paired synovial tissue samples were obtained from early RA patients by fine-needle arthroscopy as described[21]. In addition, peripheral blood and synovial tissue samples were obtained from 5 patients with early untreated RA at a second timepoint 16 weeks later.

Isolation and culture of mononuclear cells. PBMC were isolated from heparinized blood using Ficoll-Hypaque (Sigma Diagnostics, St Louis, MO, USA) density gradient centrifugation. CD4+/CD8+ T cells were isolated using biomagnetic separation, according to manufacturer's instructions (Dynabeads, Dynal, Hamburg, Germany). Total PBMC, CD4+, CD8+ and CD4–CD8– cells were pelleted, washed twice with phosphate buffered saline (PBS), and kept at –80°C until further use. To study the effect of antigen stimulation on RANK-L and OPG mRNA expression, PBMC were cultured in autologous medium [RPMI supplemented with 10% heat inactivated autologous serum, 1 mM sodium pyruvate, and 1% nonessential amino acids (Invitrogen, Merelbeke, Belgium)] at a density of 5×10^5 cells per ml. Cells were stimulated for 5 days with anti-CD3 antibody (2 µg/ml). At day 1–5, cells were pelleted in fractions of 2×10^6 cells, washed twice with PBS, and immediately frozen at –80°C until further use.

Phenotypic analysis of PBMC. Expression of cell surface markers was analyzed by flow-cytometry. Cells were resuspended in PBS with 2% fetal bovine serum and stained with phycoerythrin and/or fluorescein conjugated monoclonal antibodies specific for CD3, CD4, CD8, and CD25 (Becton-Dickinson, Erembodegem, Belgium) for 30 min at 4°C. Cells were washed twice and analyzed on a FACScan flow-cytometer (Becton-Dickinson). Phenotypic analysis was performed on anti-CD3 stimulated PBMC, prior to and after biomagnetic isolation.

Real-time PCR quantification using the LightCycler technology. Total RNA was extracted from 2×10^6 cells using the High Pure RNA isolation kit, according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany), and reverse transcribed into single-stranded cDNA using oligo-dT primer (reverse transcriptase system, Promega, Madison, WI, USA).

Primer pairs for amplification of RANK-L, OPG, T cell receptor ß constant region (TCRBC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using the computer software Oligo 6.0 (Molecular Biology Insights Inc., Cascade, CO, USA) (RANK-L F 5'-3': CTATTTCAgAgCgCAgATggAT; RANK-L R 5'-3': TATgAgAACT-TgggATTTTgATgC; OPG F 5'-3': gCTCgTgTTTCTggACATCTC; OPG R 5'-3': CCTCACACAgggTAACATCTATTC; TCR Cß F 5'-3': CCg-AggTCgCTgTgTTTgAgCCAT; TCR Cß R 5'-3': CTCTTgACCATgg-CCATC; GAPDH F 5'-3': gCTCTCCAgAACATCATCCCTgCC; GAPDH R 5'-3': CgTTgTCATACCAggAAATgAgCTT). At least one primer in all pairs spanned an exon-exon boundary that excluded genomic DNA amplification. Mononuclear cells of healthy controls were used for the initial amplification of GAPDH, TCRBC, and RANK-L, resulting in fragments of 346, 340, and 557 nucleotides, respectively. RNA extracted from the breast cancer cell line MCF-7[22] was used as a positive control for the *OPG* gene amplification reaction, which resulted in a fragment of 587 nucleotides. The resulting PCR products were cloned in a pCRII cloning vector (InVitrogen) and their sequence was confirmed by dye terminator sequence analysis. These plasmids were serially diluted (concentration range of 1×10^7 to 1×10^{-1} fg/µl) and used as relative quantification standards in the real-time PCR quantification (Roche Diagnostics, Brussels, Belgium).

PCR mixtures included 1 µM forward/reverse primer, $MgCl_2$ (3 mM for RANK-L, OPG, and TCRBC; 4 mM for GAPDH amplification reaction) and 10% LightCycler DNA master mix (Roche Diagnostics, Mannheim, Germany). PCR was performed using 45 cycles (95°C, 5 s; 55°C, 5 s; 72°C, 20 s) in the LightCycler (Roche Diagnostics, Mannheim, Germany). Annealing temperatures were 55°C, 58°C, 60°C, and 55°C for RANK-L, TCRCB, OPG, and GAPDH, respectively. In addition, PCR products were quantified using the dsDNA binding dye SYBR Green (LightCycler DNA Master SYBR Green 1, Roche Diagnostics, Mannheim, Germany). For additional specificity, the fluorescent signal was measured at a temperature between the melting point of the primer multimers and the specific PCR amplicons (84°C for RANK-L; 87°C for OPG, TCRCB, and GAPDH). The amplification products were analyzed on a 1% agarose gel. Samples were normalized by means of a housekeeping gene (*GAPDH*).

RESULTS

RANK-L and OPG mRNA levels in PBMC of patients and controls. RANK-L and OPG mRNA levels were evaluated in PBMC of patients with early and chronic RA, patients with OA, and in healthy controls by quantitative real-time PCR (Figure 1A). Mean RANK-L mRNA levels were higher for PBMC of patients with early (0.17 ± 0.07 arbitrary units, AU) and chronic RA (0.08 ± 0.03 AU) as compared to PBMC of healthy controls (0.02 ± 0.01 AU).

Table 1. Patient characteristics.

Diagnosis	Age/Sex	Disease Duration, yrs	Disease Status	Treatment
Early RA				
1	62M	<1	Stable	Pirox
2	43F	<1	Active	Pirox
3	46F	<1	Active	Pirox
4	18F	<1	Active	Pirox
5	54M	< 1	Active	Pirox
6	67F	< 1	Active	Pirox
7	46F	< 1	Active	Pirox
8	46M	< 1	Active	Pirox
9	40F	< 1	Active	Pirox
10	37F	< 1	Active	Pirox
11	60F	< 1	Stable	Pirox
12	59M	< 1	Active	Pirox
13	35M	< 1	Active	Pirox
14	68F	< 1	Active	Pirox
15	64F	< 1	Stable	Pirox
16	70M	< 1	Stable	Pirox
17	65F	< 1	Stable	Pirox
Chronic RA				
1	49F	14	Stable	Chlor, NSAID
2	64F	8	Active	MTX
3	55F	15	Stable	Chlor
4	52F	5	Active	SSZ, Chlor, MTX
5	56M	18	Active	NSAID
6	59F	20	Active	SSZ, Chlor, MTX
7	59F	14	Active	MTX
8	45F	3	Active	MTX
9	53M	6	Active	SSZ,Ridaura
10	74F	31	Active	SSZ
11	60F	12	Stable	MTX, Chlor, MP (4 mg/day)
12	61M	7	Stable	SSZ, MTX
13	68F	9	Active	MTX
14	76F	16	Stable	SSZ, Pred (5 mg/day)
Osteoarthritis				
1	75M	4		COX-2
2	76F	10		COX-2
3	69M	4		COX-2
4	72F	1		COX-2
5	55F	2		COX-2
6	63F	2		COX-2
7	65M	5		COX-2
8	65F	4		COX-2

Pirox: oral piroxicam; Chlor: chloroquine; SSZ: sulfasalazine; MTX: methotrexate; NSAID: nonsteroidal antiinflammatory drugs; COX-2: cyclooxygenase inhibitor; MP: methylprednisolone; Pred: prednisolone.

This difference was statistically significant for early as well as chronic RA patients (Student t test, $p < 0.05$). Although RANK-L mRNA levels in PBMC of OA patients (0.04 ± 0.02 AU) seemed to be higher than in healthy controls, this difference was not statistically significant. Figure 1B shows that OPG mRNA was measured in all patients with early RA, 7 of whom had high and 10 had low, but still detectable, OPG mRNA levels. Additionally, OPG mRNA was detected in 3 out of 17 healthy subjects only, and not in patients with chronic RA or OA. These data indicate an increased expression of RANK-L in blood lymphocytes of RA patients as compared to healthy controls.

RANK-L and OPG mRNA levels in CD4+ and CD8+ T cells in patients and controls. To investigate the contribution of each T cell subset to RANK-L mRNA expression in PBMC, CD4+ and CD8+ T cell subsets were isolated using biomagnetic isolation. Due to restrictions in the amount of blood available, T cell subsets were not isolated from early RA patients. Flow cytometric analysis of PBMC revealed no significant differences between the CD4+ T cell frequency

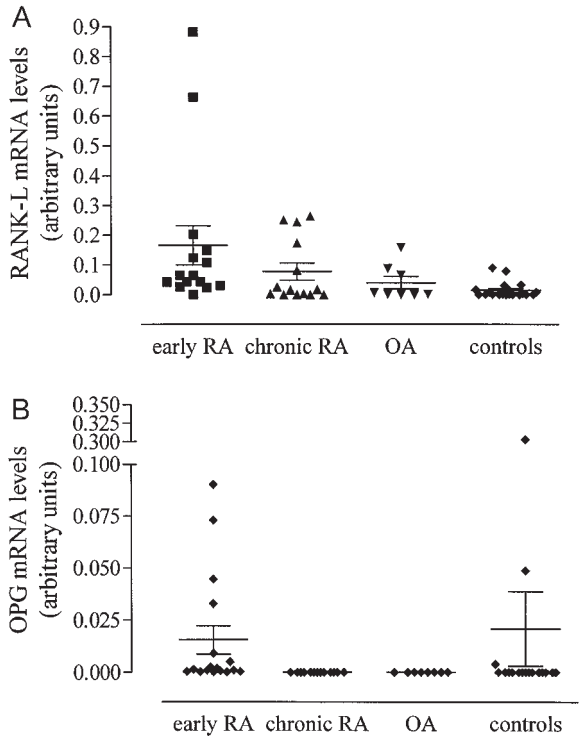

Figure 1. RANK-L (A) and OPG (B) mRNA levels in peripheral blood mononuclear cells (PBMC) of patients with early and chronic rheumatoid arthritis (RA, early: n = 17; chronic: n = 14), osteoarthritis (OA, n = 5), and controls (n = 17), as assessed by real-time PCR. Results are normalized by means of housekeeping gene *GAPDH*. Mean levels are depicted on horizontal bars; error bars indicate standard error of the mean.

in chronic RA and OA patients (50.8% ± 2.8% and 56.2% ± 6.5%, respectively) on the one hand and healthy controls (44.8% ± 2.9%) on the other hand.

As shown in Figure 2, RANK-L mRNA was detected in CD4+, CD8+, and CD4–CD8– cells. The expression profile of chronic RA and OA patients was comparable, but differed from healthy controls. Interestingly, while CD8+ T cells contributed significantly more to RANK-L mRNA levels in healthy controls, CD4+ T cells were the main contributors to RANK-L mRNA levels in RA and OA patients ($p < 0.05$). CD4–CD8– cells also showed an elevated contribution in both RA and OA patients, but this was more profound in OA patients ($p < 0.05$, compared to controls). Thus, RANK-L mRNA levels in chronic RA patients are largely of CD4+ T cell origin.

OPG and RANK-L mRNA levels in blood and synovial tissue of patients with early RA. To analyze whether mRNA levels in PBMC correlated with levels found in the joints, we compared RANK-L and OPG mRNA levels in paired PBMC and synovial tissue samples of 17 patients with early RA. The mean RANK-L mRNA levels were not significantly different for PBMC and corresponding synovial tissue samples (0.17 ± 0.07 AU and 0.07 ± 0.03 AU, respectively, data not shown). OPG mRNA was detected in

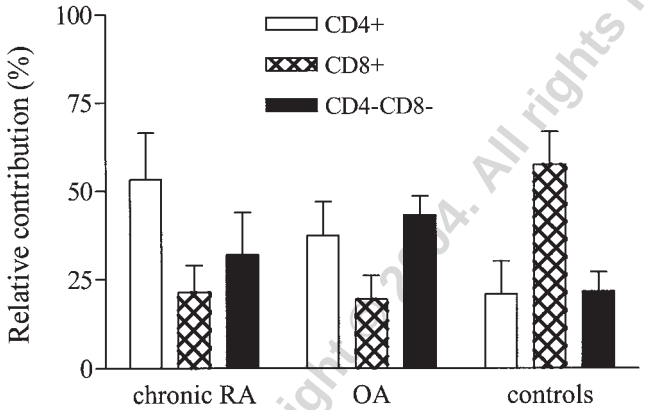

Figure 2. Relative contribution of CD4+ (white bars), CD8+ (gray bars), and CD4–CD8– (black bars) peripheral blood mononuclear cells (PBMC) to RANK-L mRNA levels in PBMC of patients with chronic rheumatoid arthritis (RA, n = 14), osteoarthritis (OA, n = 5), and controls (n = 17). Error bars indicate standard error of the mean.

synovial tissue biopsies of all early RA patients, and the expression levels tended to be higher in synovium (0.07 ± 0.04 AU) as compared to the paired blood samples (0.01 ± 0.01 AU), yet the difference did not reach statistical significance (data not shown). We analyzed the ratio of OPG/RANK-L in PBMC and synovium, since this determines the potential effects on osteoclastogenesis. Figure 3 shows that the mean OPG/RANK-L ratios were higher in synovial cells as compared to PBMC in 11 out of 17 (65%) early RA patients. No correlation was found between the OPG and RANK-L mRNA levels in blood and synovium (data not shown).

To evaluate the changes of OPG and RANK-L levels over time, mRNA levels were measured in paired peripheral blood and synovial tissue samples of 5 patients with early RA obtained with an interval of 16 weeks. Three out of 5 patients showed small changes only in OPG and/or RANK-L mRNA levels in both PBMC and synovial tissue samples

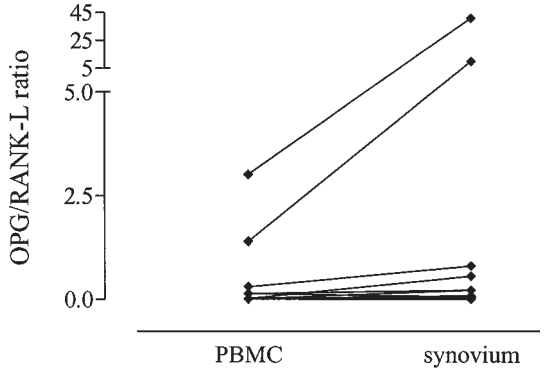

Figure 3. OPG/RANK-L ratio in paired peripheral blood mononuclear cells (PBMC) and synovial tissue samples of patients with early RA (n = 17). Mean levels are depicted on horizontal bars; error bars indicate standard error of the mean.

(patients 12, 13, and 16; Figure 4A and C). For 2 patients a relatively high increase of OPG mRNA in the synovium was found at the second timepoint, while RANK-L mRNA levels changed differently for patients 14 and 15 (Figure 4C). This resulted in an increased and decreased OPG/RANK-L ratio over time, respectively (data not shown). Moreover, these data were not consistent with the changes in PBMC, which indicates that changes in RANK-L and OPG mRNA levels in synovial tissue do not correspond with changes in PBMC.

If T cells were the main contributors to the RANK-L mRNA levels in the synovial tissue, a correlation between the levels of TCRBC mRNA and RANK-L mRNA should be found. Indeed, patients 13 and 14 showed correlating changes of RANK-L and TCRBC mRNA levels in the synovium. In the other 3 patients, however, RANK-L and TCRBC mRNA levels changed independently of each other. We also tested whether changes in RANK-L and OPG mRNA levels were correlated with clinical characteristics, but no correlations

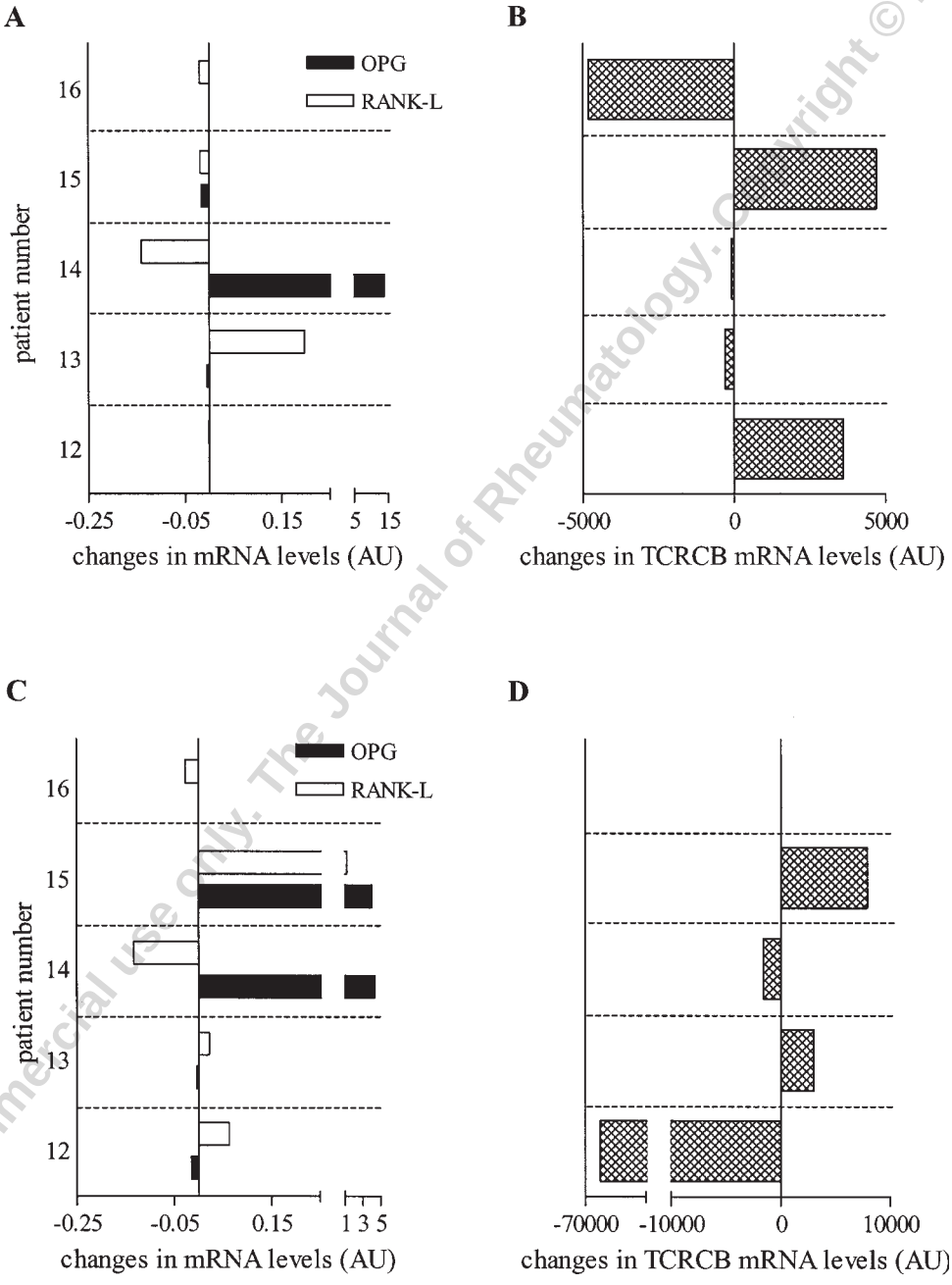

Figure 4. Changes in mRNA levels of 5 patients with early RA in 16 weeks. A. Changes in RANK-L (white bars) and OPG (black bars) levels in peripheral blood mononuclear cells (PBMC). B. Changes in T cell receptor (TCR) mRNA levels in PBMC. C. Changes in RANK-L (white bars) and OPG (black bars) levels in synovial tissue. D. Changes in TCR mRNA levels in synovial tissue. Changes were calculated as: (week 16) – (week 1).

were found (data not shown). These data indicate that short-term fluctuations in the OPG/RANK-L ratio can be found in the RA synovium, irrespective of the changes in the number of T cells in the tissue.

RANK-L mRNA expression after T cell activation. To investigate whether T cell activation may induce a temporary or longterm increase in RANK-L mRNA expression, T cells of 5 chronic RA patients and 5 healthy controls were stimulated *in vitro* for 5 days with anti-CD3 antibody, and the RANK-L mRNA levels of the cells were measured at various timepoints. As shown in Figure 5, the RANK-L mRNA levels were increased after one day of stimulation, but returned to baseline at day 2–3, suggesting that T cell activation induces a temporary upregulation of RANK-L mRNA. No differences were found between RA patients and healthy controls.

DISCUSSION

Our data show that RANK-L mRNA levels were significantly higher in PBMC of early and chronic RA patients as compared to healthy controls. Remarkably, OPG mRNA was detected in early RA patients and in 3 out of 17 controls only, but not in chronic RA or OA patients. The presence of OPG mRNA in the blood of patients with early RA, but not chronic RA and OA, suggests an attempt, although insufficient, to compensate for the elevated RANK-L mRNA levels in early untreated patients. It has been shown that glucocorticoids can suppress OPG gene transcription and enhance RANK-L levels[23]. However, only 2 of the chronic RA patients were treated with systemic glucocorticoids. This suggests that a general suppression of OPG expression occurs in later stages of the disease process independent of treatment. While some groups report no or low levels of RANK-L and OPG in healthy controls[16,24], others found lower levels of OPG in RA patients[12] or higher levels of OPG and RANK-L in RA patients[25]. These contradictory findings can be explained by different technologies used. Our data and those of others indicate that RANK-L levels are elevated in RA patients, which implies that treatment with recombinant OPG could suppress the ongoing bone destruction, especially in patients treated with corticosteroids.

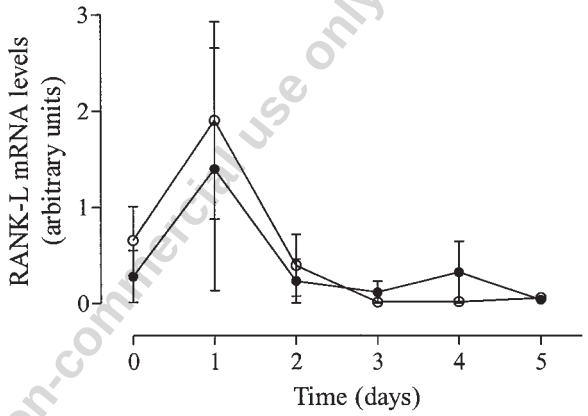

Figure 5. Effect of anti-CD3 stimulation on RANK-L mRNA expression in peripheral blood mononuclear cells (PBMC) of patients with chronic rheumatoid arthritis (RA, open circles, n = 5) and controls (black circles, n = 5). PBMC were stimulated for 5 days with anti-CD3 antibody. Cells were harvested daily and RANK-L mRNA levels were assessed by real-time PCR.

We also studied the RANK-L mRNA expression in the CD4+, CD8+ T cell subsets of chronic RA patients, OA patients, and healthy controls. The data obtained indicate that, in patients with RA but not in healthy controls, the CD4+ T cell subset contributes significantly more to the RANK-L mRNA expression than the CD8+ T cell subset. Since CD4+ T cells are thought to be actively involved in the disease process and constitute 30–50% of the synovial infiltrate[26], it is possible that CD4+ T cells, when activated, could infiltrate in the synovium and, due to their expression of RANK-L, initiate osteoclast formation and eventually lead to increased bone resorption.

No significant differences were observed between the OPG and RANK-L mRNA levels in PBMC and in the synovium of early RA patients, although the OPG mRNA levels tended to be higher in the synovium. This is in agreement with earlier studies where higher levels of both OPG and soluble RANK-L were observed in synovial fluid as compared to serum[25]. In 11 out of 17 patients the OPG:RANK-L ratio was elevated in synovium, which suggests that a compensatory mechanism might be present. This could indicate an enhanced local production of these molecules in synovium, but can also be explained by the different cell types that produce OPG and RANK-L in blood and in synovium. For these experiments, RNA was extracted from homogenized tissue biopsies. The cell composition of these lysed synovial membrane biopsies is not clearly defined. Therefore, RANK-L expression as measured in these experiments possibly originates from a mixture of cells including activated T cells, B cells, synovial fibroblasts, and even monocytes accumulating in the diseased joints. It has been shown that RANK-L expression was detected in CD4+ T cells, but also in a subset of fibroblast-like synoviocytes and monocytes of synovial tissue samples of RA patients[2,12,27]. Some of the RANK-L mRNA expression can be derived from recently activated T cells, since T cell receptor-engaged stimulation induces a transient expression of RANK-L within 24 h.

To test the contribution of T cells to the RANK-L mRNA levels in the synovium, the RANK-L mRNA and TCRBC mRNA levels were measured in synovial biopsies of untreated early RA patients 16 weeks after the first biopsy. However, no correlation between TCR and RANK-L mRNA levels was observed and changes in TCR levels did not indicate changes in RANK-L mRNA levels. This suggests that the major amount of RANK-L mRNA is of

non-T cell origin. Possible sources of RANK-L mRNA in synovial tissue are monocytes and synoviocytes. In line with this, others found that synoviocytes derived from RA patients were the main contributors to RANK-L expression[25,28]. Our results indicate that RANK-L mRNA levels in the synovium are mostly of synoviocyte origin and not of T cell origin. These findings, however, do not exclude the possibility that T cells are also involved in bone degradation in RA. Several studies have demonstrated that monocytes can differentiate into osteoclasts triggered by RANK-L expression on activated T cells[12,16].

Our data indicate that CD4+ T cells constitute 30 to 50% of the synovial infiltrate and that they are the main contributors to RANK-L expression in the mononuclear cell subset in RA patients. Thus, activated T cells that infiltrate in the RA synovium can directly initiate osteoclastogenesis in synoviocytes by the expression of RANK-L. Their production of proinflammatory cytokines such as IL-6 and TNF-α can mediate RANK-L expression on non-T cells[16,18,23]. These effects can result in elevated numbers of activated RANK-L-expressing osteoclasts, ultimately leading to bone destruction. However, due to restrictions in the amount of specimens available not all experiments were conducted in all patient groups. Nevertheless, this hypothesis is in line with the findings in adjuvant arthritis[17], which mimics many of the clinical and pathological properties of human RA. In this animal model of severe arthritis, RANK-L protein is expressed on the surface of synovial effector T cells isolated at the clinical onset of arthritis. Although inhibition of RANK-L via OPG had no effect on the severity of inflammation, OPG treatment completely abolished the loss of bone mineral density in a dose dependent manner[17]. This suggests that treatment with OPG should be combined with antiinflammatory drugs like anti-TNF-α. It is known that anti-TNF-α therapy retards but does not inhibit joint damage[29].

We analyzed the RANK-L and OPG mRNA levels by means of real-time PCR LightCycler technology[30]. This fluorescence based PCR technique allows one to monitor the accumulation of DNA using double-stranded binding SYBR Green dye. One of the major concerns in measuring mRNA expression with PCR technology is the relation with the biological activity of the protein expressed. However, it has been reported that RANK-L and OPG mRNA levels correlate well with the expression of the corresponding proteins[31]. It was also demonstrated that the ratio of RANK-L to OPG mRNA significantly correlates with the osteoclastogenic potential of synovial cells isolated from an arthritic joint[31]. Additionally, analyzing mRNA levels as an indicator of RANK-L and OPG levels in serum of RA patients instead of using protein sandwich ELISA has the advantage of avoiding false positive values due to cross-linking of the ELISA antibodies with rheumatoid factor.

We conclude that, since RANK-L and OPG mRNA levels are elevated in PBMC of RA patients, and CD4+ T cells are the major contributors to RANK-L mRNA expression, mononuclear cells in RA patients may be involved in the pathways that regulate bone metabolism. In synovial tissue, however, RANK-L mRNA is mainly of synoviocyte origin. The data suggest that these molecules are interesting targets for therapy, but additional studies are required to elucidate the role of mononuclear cells in the initiation of bone erosion in RA.

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