Osteoprotegerin (OPG)/RANK-L System in Juvenile Idiopathic Arthritis: Is There a Potential Modulating Role for OPG/RANK-L in Bone Injury?

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ABSTRACT. ***Objective.*** To evaluate serum levels of osteoprotegerin (OPG) and receptor activator of nuclear factor κB-ligand (RANK-L) in patients with juvenile idiopathic arthritis (JIA); to correlate these values with disease activity variables, radiological bone damage, and bone mass; and to correlate OPG gene polymorphisms with bone mass.

Methods. Eighty-four patients (66 girls and 18 boys) with JIA and 40 sex and age-matched controls were enrolled. Serum OPG and RANK-L were measured using an enzyme-linked immunosorbent assay. OPG genotyping was performed by polymerase chain reaction.

Results. Patients with JIA had significantly higher levels of serum OPG than controls ($p = 0.001$) and lower levels of RANK-L in comparison with controls ($p = 0.0003$). The OPG/RANK-L ratio in patients was higher than in controls ($p = 0.004$). No significant correlations were found between disease duration, erythrocyte sedimentation rate, and C-reactive protein values with either OPG or RANK-L serum levels. A significant difference in serum OPG levels (but not in RANK-L) was found between patients with and without erosions ($p = 0.008$). No correlation was found between OPG and RANK-L levels and bone mass (DXA Z scores). A higher prevalence of OPG CC genotype was found in both patients (65.4%) and controls (82.5%) ($p = 0.006$). Subjects with CC genotype had a higher lumbar spine bone mineral density (LS-BMD).

Conclusion. We evaluated for the first time levels of OPG and RANK-L in children with JIA. The higher OPG/RANK-L ratio in JIA might be the result of a compensatory production of OPG. The presence of the T allele of the OPG gene appears to be associated with low BMD. (J Rheumatol 2004;31:986–91)

Key Indexing Terms:
OSTEOPROTEGERIN
JUVENILE IDIOPATHIC ARTHRITIS
RANK-LIGAND
BONE AND CARTILAGE DAMAGE

Juvenile idiopathic arthritis (JIA) is a chronic inflammatory disease characterized by skeletal complications, the most common of which are focal bone erosions, periarticular osteopenia, and generalized reduction of bone mass[1]. Bone loss still remains a major unsolved problem in the majority of patients with JIA and can lead to fractures and decreased peak bone mass[2-5].

It has been shown that bone remodelling and homeostasis are essential in order to maintain the skeletal integrity through adult life in humans. Normal skeletal remodelling is controlled via cell-to-cell communication between osteoclasts (OC) and osteoblasts. An imbalance between bone resorption and formation results in bone loss and osteoporosis[6,7].

Recently, the role of the OC as one of the pivotal effector cells in bone resorption has been recognized[8]. It has also been shown that receptor activator of nuclear factor κB (RANK), its ligand RANK-L, and its soluble decoy receptor osteoprotegerin (OPG) are central regulators of OC recruitment and activation[8,9]. RANK-L is an essential factor for OC differentiation from monocytes and macrophages and exerts its biologic effects binding to RANK, a transmembrane tumor necrosis factor (TNF) receptor superfamily member, mainly expressed by monocyte/macrophages. In addition to its osteotropic effects, this system has important immunomodulatory functions, as evident from the phenotype of RANK-L-deficient mice, which have lymph node agenesis and thymus hypoplasia[10]. Both molecules are present in the synovial membrane of patients with rheumatoid arthritis (RA) [11].

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Submitted June 16, 2003; revision accepted December 12, 2003.

OPG, receptor antagonist to RANK, inhibits OC differentiation and activation, thus reducing bone resorption by binding RANK-L. The RANK-L/RANK/OPG system balance is therefore required to maintain bone homeostasis[10,12-15] and also represents a direct link between synovial T cell infiltration and joint and bone erosions in RA[13]. In pathological states, activated cells (e.g. infiltrating leukocytes, synovial fibroblasts) can produce molecules that shift the balance between osteoblastic and osteoclastic activities[16].

Since genetic factors have a pivotal role in determining peak bone mass[17], and OPG appears to protect from bone resorption and cartilage damage[18,19], the OPG gene may be a good candidate to identify subjects with high risk of bone loss.

To evaluate a potential role for OPG in bone damage of JIA, we measured serum and synovial fluid OPG and RANK-L levels in a cohort of patients with JIA, and correlated them with erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), disease duration, bone erosions, and bone mineral density (BMD).

In addition, a polymorphism of OPG gene in intron 2 has been studied in the present population, and the association with BMD was examined.

MATERIALS AND METHODS

Clinical characteristics of study population. Our study population included 84 patients (66 girls and 18 boys) with a median age of 8 years (range 5.6-11.4 yrs), and a mean disease duration ± SEM of 3.6 ± 2.4 years (range 1.2-6.1 yrs) who were classified with JIA according to the Durban criteria[20]. Forty-six had polyarticular, rheumatoid factor negative disease and 38 had oligoarticular onset disease; 5 out of 38 patients with oligoarticular disease had extended oligoarthritis. At the time of the study all children had active disease (joint swelling with pain and reduced motion in one or more joints). With regard to therapy, all patients with polyarthritis and patients with extended oligoarthritis were receiving methotrexate and nonsteroidal anti-inflammatory drugs (NSAID); patients with oligoarthritis were treated with NSAID only. No patients were receiving or had received oral or intraarticular corticosteroids prior to study onset.

Forty sex and age-matched healthy children attending our outpatient clinic for musculoskeletal pain without signs of inflammation were studied as controls after the exclusion of rheumatic, endocrine, or metabolic diseases. All patients and controls were prepubertal. Approval was obtained by the Ethics Committee of Meyer Hospital and parents or guardians gave informed consent.

Bone erosions. Conventional radiographs of clinically involved joints were reviewed for bone erosions.

BMD. Bone status was evaluated at the time of the study by the same physician (LM) in all patients using dual x-ray absorptiometry (DXA) (Hologic QDR 1000/W, Waltham, MA, USA) at the lumbar spine (L2-L4, postero-anterior).

As no consensus criteria defining osteopenia and osteoporosis on the basis of BMD measurements have been established in children, for this study we set a cut-off point for osteopenia at a BMD Z score of less than –1.5.

Serum and synovial fluid assays. Sera were obtained by centrifuging blood collected by venipuncture during routine laboratory tests. Synovial fluid was collected during arthrocentesis before intraarticular steroid injection.

OPG and RANK-L concentrations were determined in serum and in synovial fluid using a highly sensitive, commercial sandwich enzyme immunoassay provided by Immundiagnostik (Bensheim, Germany). Measurements were performed in samples according to the manufacturer's instructions. The lower limit of detection of this assay is 2.8 pg/ml for OPG and 8 pg/ml for RANK-L. The intraassay and interassay (n = 16) coefficient of variation (CV) is < 10% for OPG. The intraassay (n = 16) CV is between 5-7% and interassay (n = 10) CV is 7-9% for RANK-L. All assays were measured blinded to any clinical information. Each experiment was performed in duplicate.

Genotyping. After informed consent was obtained, blood samples were drawn during routine followup laboratory tests. Genomic DNA was isolated from EDTA-treated blood samples by a standard phenol-chloroform extraction procedure.

For OPG polymorphism, genomic DNA was amplified by polymerase chain reaction (PCR) as indicated by Wuyts, *et al*[21]. PCR was carried out as end products were digested by Pst I enzyme (New England Biolabs, Beverly, MA, USA) under conditions recommended by manufacturer and electrophoresed in 3% NuSieve and 1% Agarose. Pst I restriction site was introduced in the presence of T allele. The presence or absence of the restriction site was indicated as TT and CC, respectively. The heterozygous were indicated as TC.

Statistical analysis. Values of OPG and RANK-L were expressed in pg/ml as mean ± SEM. Mann-Whitney U test and analysis of covariance (ANCOVA) with least significant difference (LSD) correction were used to evaluate the differences of OPG and RANK-L between groups. Wilcoxon signed rank test was applied to detect differences between synovial and serum assays. The Spearman rank correlation test was used to determine correlation coefficients between OPG and RANK-L levels and disease duration, ESR, and CRP values.

For the assessment of OPG polymorphism distribution in the total population, Pearson's chi-square analysis was applied (odds ratios, OR, with 95% confidence intervals, CI). ANCOVA followed by LSD to protect for least significant difference, was performed in order to evaluate the correlation between OPG polymorphisms and LS-BMD values, presented as means ± SEM. The following covariates were considered for the ANCOVA: sex, age, pubertal stage, BMI, and disease duration. Nonparametric tests were used, where necessary, in univariate analysis due to the small size of our groups and to the skewness of our data. Levels of $p < 0.05$ were considered statistically significant. Statistica 5.1 (Statsoft Inc., Tulsa, OK, USA) was the software used.

RESULTS

The mean concentration of serum OPG in healthy subjects was 39.6 ± 11 pg/ml and the mean concentration of serum RANK-L was 128.17 ± 39.3 pg/ml.

Patients with JIA had significantly higher levels of serum OPG than controls (60.19 ± 15 pg/ml vs 39.6 ± 11 pg/ml; $p = 0.001$). Patients with polyarticular and extended oligoarticular disease had higher serum levels of OPG in comparison with patients with oligoarticular disease ($p = 0.001$) and controls ($p = 0.016$) (Figure 1). No significant differences in OPG levels were observed between patients with oligoarticular onset JIA and controls. Furthermore, no significant correlation was found between serum OPG and ESR, CRP, or disease duration.

Bone erosions were detected radiologically in 29 out of 84 patients, 24 with polyarticular onset and 5 with extended oligoarthritis. Patients with radiographic erosions showed significantly higher concentrations of serum OPG than patients without radiographic erosions ($p = 0.007$) (Figure

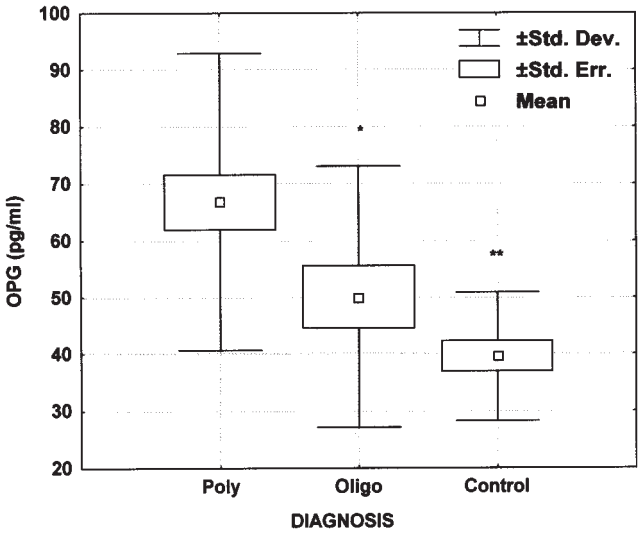

Figure 1. Differences in the serum OPG levels among patients with polyarticular and oligo-extended (poly), oligoarticular (oligo) disease, and controls. ANCOVA followed by LSD test showed that patients with poly-disease had higher serum levels of OPG in comparison with subjects with oligo disease (*p = 0.001) and with controls (**p = 0.01). No statistically significant differences were observed between oligo and control groups.

2). No correlation was found between serum OPG levels and DXA Z score values.

Synovial fluid was available from 10 patients. Mean synovial OPG levels were slightly higher than serum OPG obtained from the same patients, but the difference did not reach statistical significance (p = 0.3).

In addition, patients with JIA had lower levels of RANK-L in comparison with controls (86.57 ± 12 pg/ml vs 128.17 ± 39.3 pg/ml; p = 0.0003). Lower values than controls were also seen in the different disease onset groups: polyarticular and extended oligoarticular onset (p = 0.0004); oligoarticular onset (p = 0.001) (Figure 3). No significant differences in the levels of RANK-L were observed between patients with polyarticular and extended oligoarticular disease and patients with oligoarticular disease.

No significant correlation was found between serum RANK-L, and ESR, CRP, disease duration, bone erosions, and DXA Z score values. Mean synovial RANK-L levels were slightly higher than serum RANK-L obtained from the same patients, even though this did not reach a statistically significant value (p = 0.6). Finally the OPG/RANK-L ratio was 0.31 ± 0.4 in controls and 0.61 ± 0.7 in patients (p = 0.004).

Genotyping. Pearson's chi-square analysis showed a prevalence of CC genotype for OPG gene in the total population (70.9%), in JIA (65.4%), and in controls (82.5%). The subjects were Caucasian and the frequency distribution of the genotypes were in Hardy-Weinberg equilibrium. In addition, considering the prevalence of the different genotypes in JIA versus controls, the TC genotype was more frequent in JIA (86% vs 14%, Pearson's chi-square test: 8.9; p = 0.006) (Table 1). Applying ANCOVA and LSD test to evaluate the LS-BMD differences according to the OPG genotypes in the total population (124 subjects), we observed that patients with CC genotype had a higher LS-BMD in comparison with the TT genotype (p = 0.03) and the TC genotype (p = 0.02) (Figure 4). The same findings were detected considering JIA patients: CC genotype had a statistically significant higher BMD at the lumbar spine in

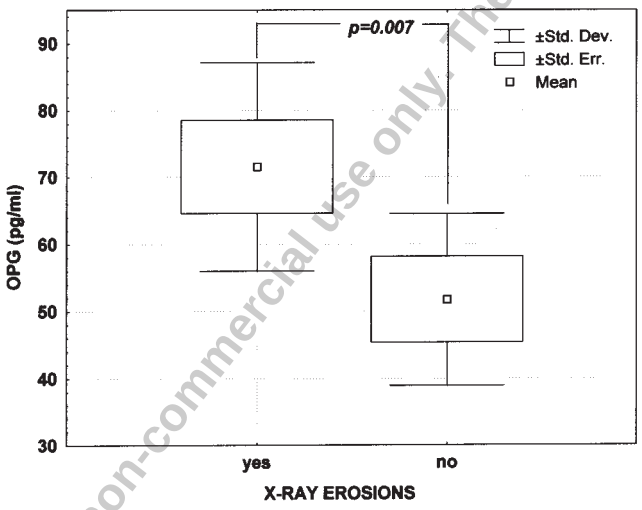

Figure 2. Serum OPG levels in patients with and without erosions evaluated by radiographs. Patients with erosions (yes) showed higher levels of serum OPG in comparison with patients without erosions (no). Mann-Whitney U test: p = 0.007.

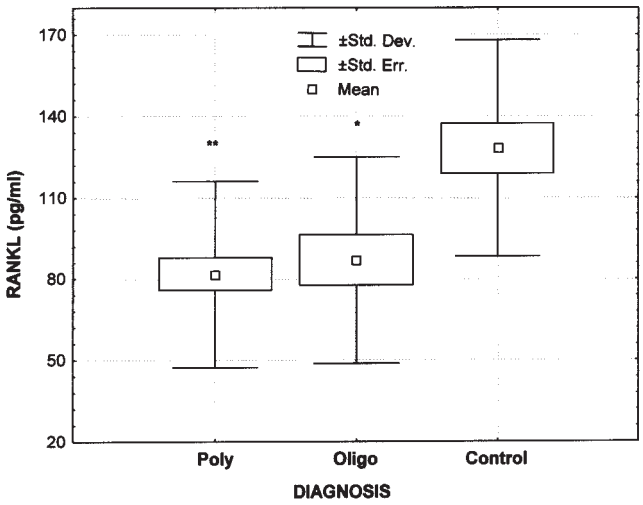

Figure 3. Differences in the serum RANK-L levels among patients with polyarticular and oligo-extended (poly), oligoarticular (oligo) disease, and controls. ANCOVA followed by LSD test showed that patients with poly and oligo disease had lower serum levels of RANK-L in comparison with control subjects (**p = 0.0004 and *p = 0.001). No statistically significant differences were observed between poly and oligo subjects.

Table 1. Distribution of OPG genotypes in the studied population, expressed as number (%) of positive subjects.

Genotype	JIA, No. of Observed (%)	Controls, No. of Observed (%)	Total Population, No. of Observed (%)
CC	55	33	88
Row percent	(62.5)	(37.5)	
Column percent	(65.4)	(82.5)	
Total percent			(70.9)*
TT	4	3	7
Row percent	(57)	(43)	
Column percent	(4.8)	(7.5)	
Total percent			(5.7)
TC	25	4	29
Row percent	(86)**	(14)	
Column percent	(29.8)	(10)	
Total percent			(23.4)

* Pearson's chi-square analysis showed a prevalence of CC genotype for OPG gene in the total population, in JIA and in controls. ** TC genotype was more frequent in JIA than in controls. Pearson's chi-square test: 8.9; p = 0.006.

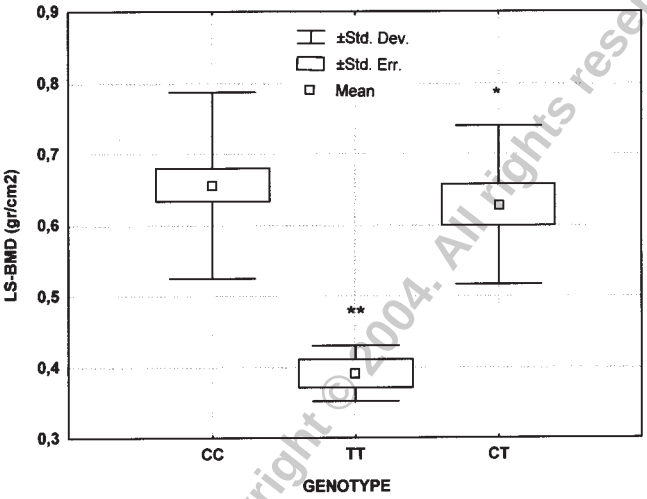

Figure 5. Differences in the LS-BMD in patients with JIA with various OPG genotypes. ANCOVA and LSD test: subjects with the CC genotype had a statistically significant higher LS-BMD in comparison with the TT (**) genotype (p = 0.02) and the TC (*) genotype (p = 0.04).

comparison with the TT genotype (p = 0.02) and the TC genotype (p = 0.04) (Figure 5).

Finally, no significant differences in levels of serum OPG were found between the 3 genotypes (data not shown).

DISCUSSION

We evaluated for the first time the amount of OPG and RANK-L in the serum of children with chronic arthritis. Significantly higher levels of serum OPG were observed in subjects with JIA, and in particular in patients with polyarticular and extended oligoarticular disease than patients with oligoarticular disease or healthy controls. The production of OPG and its ligand is regulated by various proinflammatory cytokines known to affect bone metabolism[22]; *in vivo* studies in animal models of RA showed that activated T-lymphocytes that produce RANK-L have been implicated in the stimulation of OC and promotion of bone loss[23]. We could speculate that patients with more severe disease could have higher levels of serum OPG due to a compensatory self-defence response for keeping under control immune mechanisms responsible of bone and cartilage destruction. A similar interpretation was made by Hofbauer, *et al*[24], who found a link between high serum concentrations of a presumptive vascular protective factor (OPG) and a high prevalence of cardiovascular disease. Another explanation could be that higher OPG levels could be secondary to a decreased clearance. In fact, the amount of RANK-L was lower in patients affected by JIA in comparison with controls. In addition, the serum RANK-L test kit is an enzyme immunoassay designed to determine soluble, uncomplexed RANK-L; it is possible that part of the molecule could bind to its receptor and part to the OPG so that free RANK-L is less available in the serum.

Finally, OPG/RANK-L ratio is a crucial determinant of OC differentiation and activation in RA[25]. In this study, the OPG/RANK-L ratio was higher in subjects with JIA than in controls. This agrees with the result of a higher compensatory production of OPG, which bind RANK-L to contrast the damage of RANK-L-RANK complex on bone and cartilage.

With regard to variables of disease activity or severity, we did not find any significant correlation between serum OPG and ESR, CRP, or duration of the disease. However, serum OPG was higher in patients with radiographic

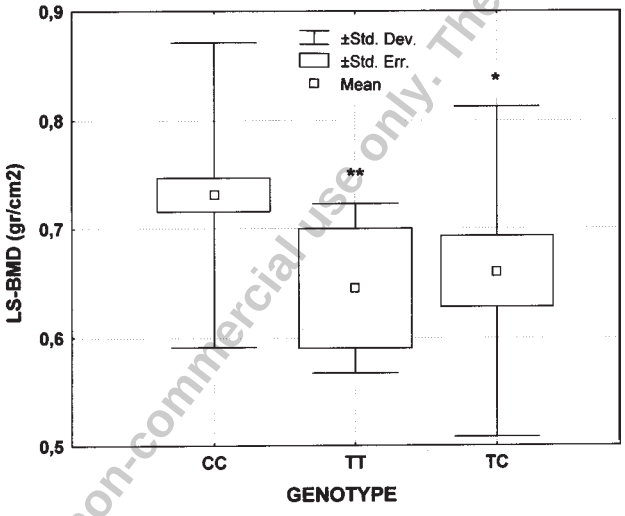

Figure 4. Differences in the LS-BMD in total population with various OPG genotypes. ANCOVA and LSD test: subjects with CC genotype had a statistically significant higher LS-BMD in comparison with the TT (**) genotype (p = 0.03) and the TC (*)genotype (p = 0.02).

erosions. It is likely that local cytokines such as interleukin (IL)-1, TNF, IL-17, IL-11, known to be abundant in inflamed synovial tissues in JIA and important modulators of bone and cartilage erosion could also have a crucial role in the stimulation of OPG production. These data are supported by observations showing a pivotal role of OPG and RANK-L derived from various cells in the rheumatoid synovial environment in the formation of marginal erosions[19]. In Lewis rats with adjuvant-induced arthritis, OPG treatment completely blocks the loss of BMD and bone erosion, and the preservation of bone by OPG is associated with conservation of the articular cartilage matrix[23,26]. Higher amounts of OPG were found in the synovial fluid of our patients, although the difference in the OPG serum levels did not reach statistical significance, most likely because of the low number of samples available.

We found no significant correlations between serum OPG and levels of BMD as evaluated by DXA. These data are in agreement with those from Browner, *et al*[27] and in contrast to those of Yano, *et al*[28] and Arrighi, *et al*[29]. However, there are substantial differences between these studies and ours, mostly in that their findings were derived from adult and not pediatric populations, where several factors could modify the results. In addition, BMD is critically determined by a variety of genetic and environmental factors and not by a single cytokine. Expression of OPG in transgenic mice and administration of recombinant protein into normal mice resulted in nonlethal osteopetrosis associated with decreased OC[30]. OPG knock-out mice developed by Bucay, *et al*[31] exhibited a decrease in total bone density and a high incidence of fractures due to enhanced osteoclastogenesis. These findings suggest that the OPG gene would be an excellent candidate gene for an investigation into susceptibility to osteoporosis in humans. We found a positive association between particular OPG gene polymorphisms and BMD. In particular, the presence of the T allele identified in the intron 2 of the OPG gene appeared to have a significant association with low BMD phenotype in children. Subjects with the TT genotype had a BMD 23.9% lower than those with the opposite genotype (CC); heterozygous subjects (TC) had intermediate BMD values.

Considering patients only, we observed that subjects with the TT genotype had a BMD 40% lower than those with the CC genotype and 36% than the TC genotype. These data are in agreement with those in the literature that show a major frequency of the T allele in patients with Paget's disease of bone[21]. This sequence variation is an intronic polymorphism close to the splice junction of exon 3. We speculate that the presence of the T allele might modify the splicing efficiency. A linkage disequilibrium between intron 2 polymorphic site and another polymorphism in the promoter region might explain our findings, as suggested by the presence of polymorphic sites in the promoter region of the OPG gene associated with low BMD at the lumbar spine in postmenopausal women[32]. Finally, a recently published study by Langdahl, *et al* showed a T^{245}-G polymorphism localized in the promoter region; the authors found an association of this polymorphic site with an increase of fracture risk in adult population[33]. Further investigations are in progress to evaluate this aspect in our population.

In conclusion, we found significant differences in the levels of OPG and RANK-L in children with JIA when compared with healthy controls. The increase of serum OPG in patients with severe disease could reflect a compensatory response to degeneration of bone and cartilage. An OPG polymorphism may represent a marker in the identification of patients with a higher risk of losing bone mass.

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