The HLA-B27 Transgenic Rat, a Model of Spondyloarthritis, Has Decreased Bone Mineral Density and Increased RANKL to Osteoprotegerin mRNA Ratio

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ABSTRACT. ***Objective***. Bone metabolism in spondyloarthritis (SpA) is not well elucidated. We investigated alterations in bone in the HLA-B27 transgenic rat, a model of SpA.

Methods. Femur, tibia, and lumbar vertebral bodies of disease-prone HLA-B27 transgenic, healthy HLA-B7 transgenic, and nontransgenic control rats were used for bone histomorphometric and dual energy x-ray absorptiometry (DEXA) analysis. Serum levels of type I collagen C-telopeptides (CTX), N-terminal propeptide of type I procollagen (P1NP), and osteocalcin, as well as receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG), were measured. RNA was isolated from the bone tissue of the femura to analyze gene expression of RANKL, OPG, and osteocalcin.

Results. Histomorphometric analysis indicated a significant decrease in bone volume as well as trabecular number and thickness in the HLA-B27 rats. Trabecular separation was increased. Numbers of osteoblasts, osteoclasts, and osteoid volume were not altered significantly. The decrease in bone mineral density was confirmed using DEXA. Levels of RANKL mRNA were significantly increased in the bone tissue of HLA-B27 transgenic rats, resulting in an increased RANKL to OPG ratio. Osteocalcin mRNA expression was also significantly elevated in bone of HLA-B27 rats. Serum levels of CTX, RANKL, OPG, P1NP, and osteocalcin did not differ significantly.

Conclusion. Our data indicate that, similarly to SpA in humans, HLA-B27 transgenic rats show a reduced bone mass, and suggest an involvement of the RANKL/OPG system in the mechanism of bone loss in this disease. This model may be adequate to study osteoporosis in SpA. (First Release Dec 1 2008; J Rheumatol 2009;36:120–6; doi:10.3899/jrheum.080475)

Key Indexing Terms:
SPONDYLOARTHROPATHY HLA-B27 TRANSGENIC RAT BONE
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Spondyloarthritis (SpA) is a common variety of chronic inflammatory disease characterized by the predominance of axial joint inflammation, frequent peripheral arthritis and enthesitis, and extraarticular features, such as anterior uveitis, psoriasis and inflammatory bowel disease (IBD)[1]. In addition to the formation of new bone leading to syndesmophytes and ankylosis, which is a major characteristic of ankylosing spondylitis (AS), the prototypical form of SpA, osteoporosis is an often reported feature as well[2]. Thus, this disorder raises the challenging paradox of new bone formation at sites of inflammation coexisting with reduced bone mass and increased fracture risk.

The exact etiology of SpA remains largely unknown. Nevertheless, genetic predisposition to SpA is strong and mainly dominated by its association with the major histocompatibility complex class I molecule HLA-B27[3]. The introduction of the HLA-B27 and human ß$_2$-microglobulin (h-ß$_2$m) transgenes into rats leads to spontaneous development of a multisystem disease including peripheral arthritis, gastrointestinal inflammation, and inflammatory skin lesions.

Depending on the genetic background of the rats spondylitis and intervertebral discitis may also occur[4]. Moreover, this disease is specific for HLA-B27, since rats transgenic for the control HLA-B7 allele, which is not associated with SpA in humans, and h-ß$_2$m remain healthy[4].

Investigations of bone metabolism in this model remain scarce. Nevertheless, an accelerated alveolar bone loss and reduced bone strength due to impaired bone microarchitecture have been described[5,6]. Bone metabolism clearly appears unbalanced, although the cellular and molecular mechanisms leading to bone loss have not been elucidated. Receptor activator of nuclear factor-κB ligand (RANKL) has been identified as a potent osteoclast-stimulating cytokine, whereas osteoprotegerin (OPG) acts as its decoy receptor, inhibiting its actions[7]. Dysregulations in the RANKL/OPG system have been implicated in various bone-loss diseases including osteoporosis, rheumatoid arthritis, and multiple myeloma[8]. In patients with AS, serum concentrations of RANKL have been found to be elevated, and we previously showed an increased expression of intracellular RANKL in CD4+ and CD8+ T cells from patients with AS[9]. In contrast, serum levels of OPG remain controversial in patients with AS showing increased, decreased, or unchanged levels[10-12]. However, there are no reports on the regulation of the RANKL/OPG system in the HLA-B27 transgenic rat model.

Although new bone formation at sites of peripheral arthritis is another common feature of AS and is also seen in HLA-B27 transgenic rats, no systemic studies of bone formation markers have been conducted in this model[4]. In humans, serum levels of bone formation markers in patients with AS are still a matter of debate. While 2 studies have demonstrated increased levels of osteocalcin in patients with AS[11,13], 2 other reports showed that serum osteocalcin is decreased[14,15]. Yilmaz and Ozaslan found no significant changes in levels of the bone formation markers osteocalcin and bone-specific alkaline phosphatase[16].

We investigated the structural and cellular changes in the bone tissue of HLA-B27 transgenic rats by bone histomorphometry, and assessed alterations in the RANKL/OPG system in this disease model. This is the first study to examine serum and mRNA levels of bone formation markers in HLA-B27 transgenic rats.

MATERIALS AND METHODS

Disease-prone HLA-B27/h-ß$_2$m transgenic rats of the 33-3 line on a Fischer (F344) background, their nontransgenic littermate controls, and healthy homozygous HLA-B7/h-ß$_2$m transgenic rats of the 120-4 line backcrossed to an F344 background were used in this study, as described[17]. Features include arthritis, colitis, and inflammatory skin lesions as well as occasional occurrence of spondylitis. These lines, originally produced at the University of Texas Southwestern Medical Center[18], have been continuously bred and maintained at the Cochin Hospital, Paris, for several years (animals were a kind gift from Dr. J. Taurog, Dallas, TX, USA). Six rats per group, matched for sex (female/male ratio 4/2) and age (6–20 months old), were used in all experiments. Study procedures were approved by the institutional animal care committee of the Institut Cochin.

Bone histomorphometry. Femur, tibia, and the fourth lumbar vertebral body of the lumbar spine of HLA-B27 and HLA-B7 transgenic rats and nontransgenic controls were fixed in formalin and dehydrated in an ascending ethanol series. Then the bones were embedded in methylmethacrylate and cut into 4-µm sections. After mounting, the sections were stained with Goldner's trichrome stain. Bone volume per total volume (BV/TV), bone surface per total volume (BS/TV), bone surface per bone volume (BS/BV), trabecular number (Tb.N), trabecular separation (Tb.Sp), number of osteoblasts (Ob.N), number of osteoclasts (Oc.N), and osteoid volume per total volume (OV/TV) were measured with the Osteomeasure system (OsteoMetrics, Decatur, GA, USA). The measurements, terminology, and units used for histomorphometric analysis were those recommended by the Nomenclature Committee of the American Society of Bone and Mineral Research[19].

Dual energy x-ray absorptiometry (DEXA). Bone mineral density (BMD) of the femur, tibia, and sixth lumbar vertebral body were analyzed by DEXA using the Lunar iDXA bone densitometer and the enCORE "hand" software (GE Healthcare Corp., Munich, Germany). Bones from each animal were removed and preserved in 70% ethanol until measurement.

Serum measurements. Serum levels of free soluble RANKL and OPG (Biomedica, Vienna, Austria), type I collagen cross-linked C-telopeptide (CTX; RatLaps, IASON, Graz, Austria), N-terminal propeptide of type I procollagen (P1NP)[20], and osteocalcin (N-mid osteocalcin; IDS, Frankfurt, Germany) were analyzed with a commercial ELISA.

Gene expression analysis. RNA was extracted by crushing the long bones in liquid nitrogen and proceeding with the Trizol method following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Freshly isolated RNA was dissolved in 0.01% RNase-free water and the concentration was measured at 260 nm using a spectrophotometer. Purity was assessed by the A260/280 ratio and RNA integrity was confirmed on a 1% agarose gel. 2 µg RNA were reverse-transcribed into cDNA (Superscript II First-Strand; Invitrogen) and used for quantitative real-time polymerase chain reaction (RT-PCR). RT-PCR was performed for RANKL, OPG, and osteocalcin using assay-on-demand probes (Applied Biosystems, Foster City, CA, USA). The analyses were performed on the ABI Prism 7700 Sequence Detection System (Applied Biosystems). All experiments were performed in duplicates and were normalized to an invariant endogenous control (GAPDH). PCR conditions were 50˚C for 2 min and 94˚C for 2 min followed by 40 cycles at 94˚C for 15 s and 60˚C for 30 s. Results were calculated applying the ∅∅CT method and are presented as fold-increase relative to GAPDH expression.

Statistical analysis. Statistical analyses were performed using Student's t test. Results are presented as means and standard deviations (SD) or median and range. p values < 0.05 were considered statistically significant.

RESULTS

Bone histomorphometry was conducted to assess the histological features of bone tissue from HLA-B27 and HLA-B7 transgenic rats and their nontransgenic F344 littermates. Femur, tibia, and the fourth lumbar vertebral body of the HLA-B27 transgenic rats exhibited a significant reduction of BV/TV (Figure 1A, 1E) and BS/TV (Figure 1C), as compared to HLA-B7 transgenic and nontransgenic rats. The resulting BS/BV ratio (Figure 1D) showed a significant increase in HLA-B27 transgenic rats. In addition to the reduced bone volume, BMD was also reduced in the femur of HLA-B27 transgenic rats, as measured by DEXA. However, a nonsignificant decline in BMD was seen in the tibia and sixth vertebral body (Figure 1B). Moreover, trabecular thickness and number were significantly reduced in the diseased rats, while trabecular separation was significantly increased (Figure 2A). In all 3 groups of rats, the number of osteoblasts and osteoid volume

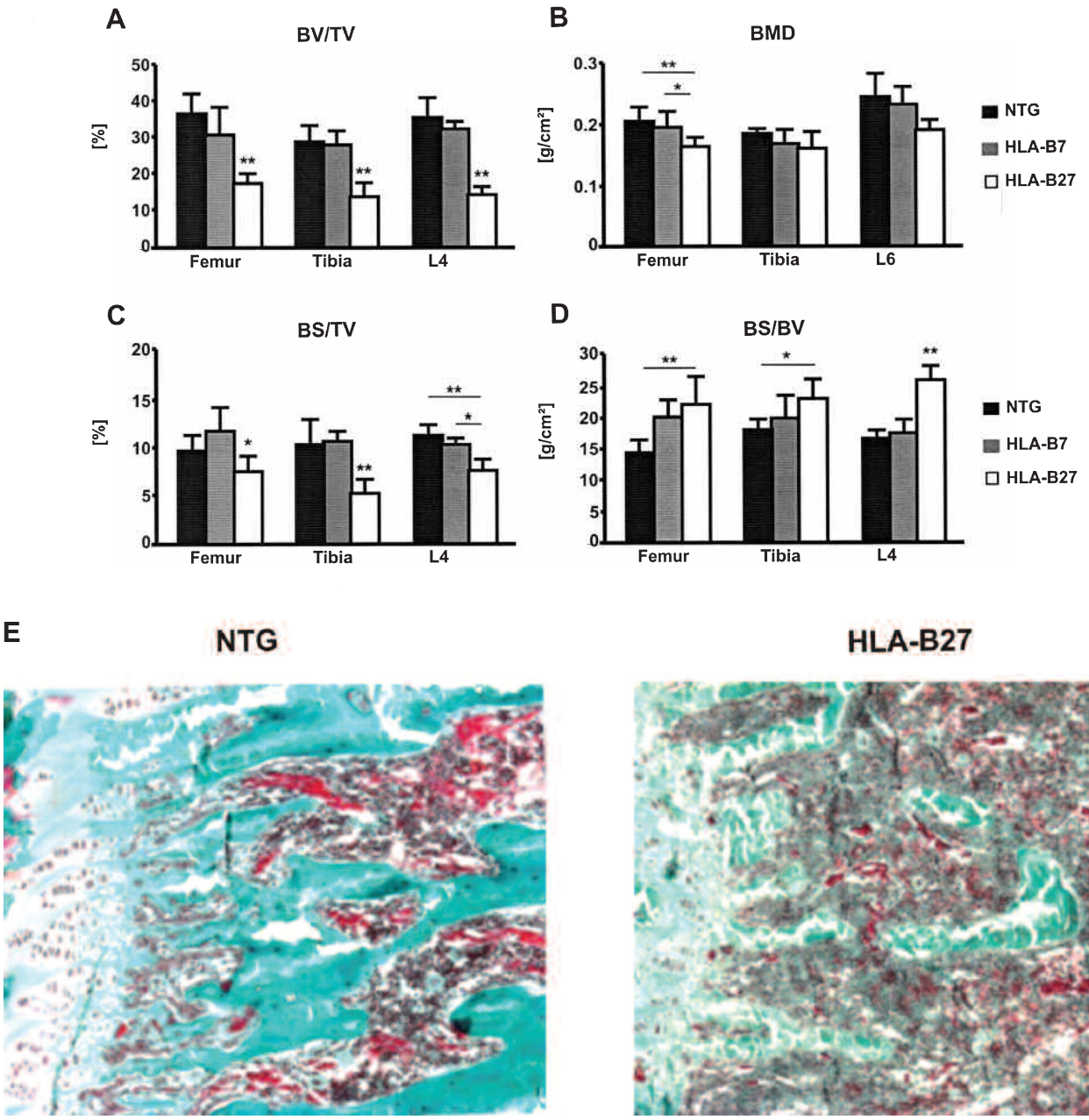

Figure 1. Decreased bone mass in HLA-B27 transgenic rats. Histomorphometric analysis showed a decreased bone volume (A) and bone surface (C) and increased bone surface related to bone volume (D) in HLA-B27 transgenic rats compared to nontransgenic (NTG) and HLA-B7 controls. (B) Lower bone mass was also confirmed by DEXA. Each bar represents the mean + SD per group. *p ≤ 0.05, **p ≤ 0.01; p values show significance of HLA-B27 versus nontransgenic and HLA-B7 controls if not indicated otherwise. (E) Representative histological sections stained with Goldner's trichrome show markedly reduced mineralized bone volume (turquoise color) in the HLA-B27 transgenic rats compared to nontransgenic rats (original magnification x20).

were higher in femur and tibia than in the vertebral body; however, these measures did not show any variation between the groups (Figure 2B). Osteoclasts tended to be more abundant in the HLA-B27 transgenic rats, although the difference was not statistically significant versus any of the controls (Figure 2B).

Although the data obtained by bone histomorphometry consistently demonstrated reduced bone mass in HLA-B27 transgenic rats, serum levels of CTX were not increased in the diseased rats (Figure 3). Because the RANKL/OPG system has often been found to be disturbed in diseases with bone loss, we investigated whether alterations in the RANKL/OPG ratio were associated with the bone loss in the HLA-B27 transgenic rats. Whereas no differences were found in serum levels of RANKL and OPG, RANKL mRNA levels in bone increased significantly in HLA-B27 transgenic rats compared

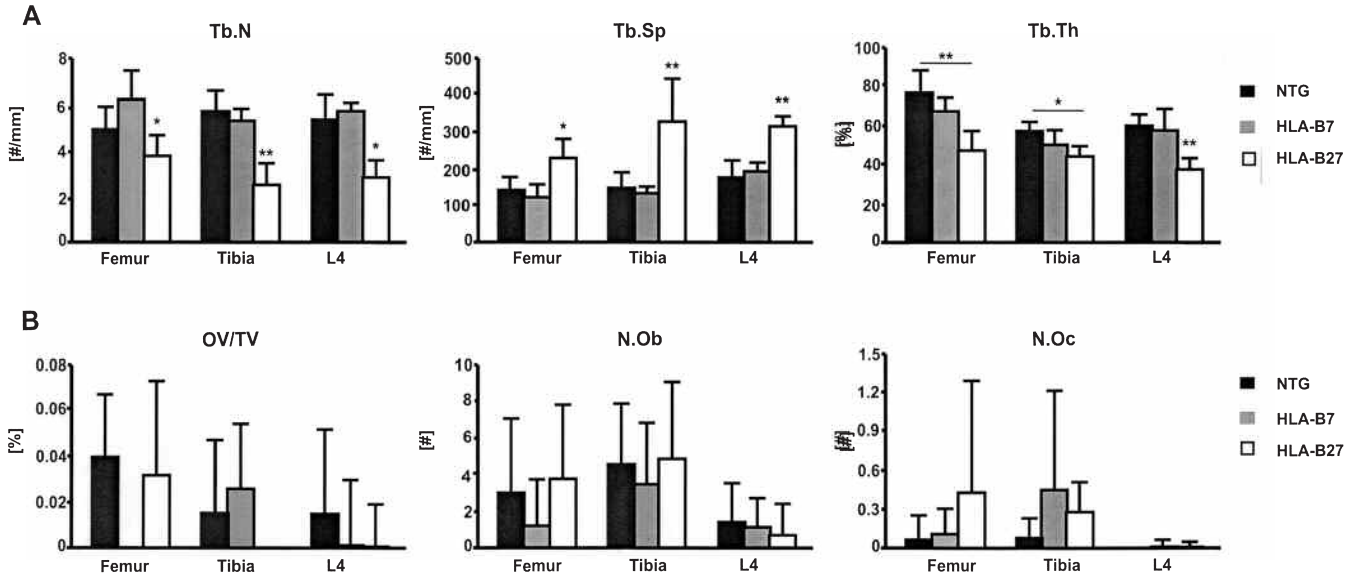

Figure 2. HLA-B27 transgenic rats show alterations in structural indices. (A) Histomorphometric analysis showed decreased trabecular number (Tb.N) and trabecular thickness (Tb.Th) and increased trabecular separation (Tb.Sp) in femur, tibia, and L4 of the HLA-B27 transgenic rats. (B) Osteoid volume (OV), number of osteoblasts (N.Ob), and number of osteoclasts (N.Oc) showed no alterations. Each bar represents the mean + SD per group. *p ≤ 0.05, **p ≤ 0.01; p values show significance of HLA-B27 versus nontransgenic (NTG) and HLA-B7 controls if not indicated otherwise. TV: total volume.

to the HLA-B7 and the nontransgenic control groups. Although mRNA levels of OPG did not differ in bone, the RANKL to OPG ratio was significantly increased in the HLA-B7 and HLA-B27 transgenic rats compared to the nontransgenic control group (Figure 3). However, no statistical difference was found in the RANKL to OPG ratio between HLA-B7 and HLA-B27 transgenic rats.

Due to the appearance of syndesmophytes in the spine in aging HLA-B27 transgenic rats[21], and reports of osteophytes along the diaphyses as well as foci of metaplastic bone within the fibrotic joint capsules[22], investigations of systemic markers of bone formation were undertaken. Serum levels of osteocalcin and P1NP were determined; no statistical difference was found between the groups (Figure 4). Additionally, osteocalcin mRNA levels were investigated in the bone tissue to assess the local expression of a bone formation marker. Osteocalcin mRNA expression in bone was significantly elevated in the HLA-B7 and HLA-B27 transgenic rats compared to the nontransgenic group (Figure 4). The increase in osteocalcin expression in the HLA-B27 transgenic rats compared to the HLA-B7 transgenic rats just failed to reach statistical significance ($p < 0.052$).

DISCUSSION

Although the HLA-B27 transgenic rat model of SpA, existing since the early 1990s, is a well studied animal model of inflammatory cutaneous and intestinal conditions as well as peripheral arthritis, there has been little effort to investigate alterations in bone metabolism. The first study of bone metabolism in the jaw of HLA-B27 transgenic rats, by May and Tatakis, showed an accelerated alveolar bone loss[5]. In a recent study, Akhter and Jung found that bone microarchitecture evaluated by micro-computed tomography analysis and bone strength measured by a 3-point bending test were significantly decreased in HLA-B27 transgenic rats[6]. These studies focused mainly on microarchitectural and biomechanical properties of bone, but did not provide insight into the histopathological and molecular processes.

Using bone histomorphometry we found that bone mass was reduced in HLA-B27 transgenic rats, a result that is fully consistent with the previous reports. This decrease was mainly reflected by alterations in the trabecular structure. Moreover, we found that the numbers of osteoblasts and the osteoid volume were not different among the 3 study groups. The finding that osteoid volume was not changed excludes mineralization defects possibly occurring due to calcium and vitamin D malabsorption as a consequence of the chronic IBD in the HLA-B27 transgenic rats. However, since the gut inflammation is a source of various proinflammatory cytokines that may contribute to bone destruction, the role of IBD in the pathogenesis of bone loss in these rats still remains to be investigated. A good model to do this would be the recently described rat line 21-3x283-2, which displays spondylitis and arthritis in males but does not lead to IBD[23]. Finally, although male HLA-B27 transgenic rats often display orchitis or epididymitis, the resulting hormone dysregulation does not seem to contribute significantly to the bone destruction, since female rats showed a similar reduction in bone mass[2].

Since the RANKL/OPG/RANK pathway has often been shown to mediate bone resorption in various inflammatory bone diseases including SpA, we investigated the participation

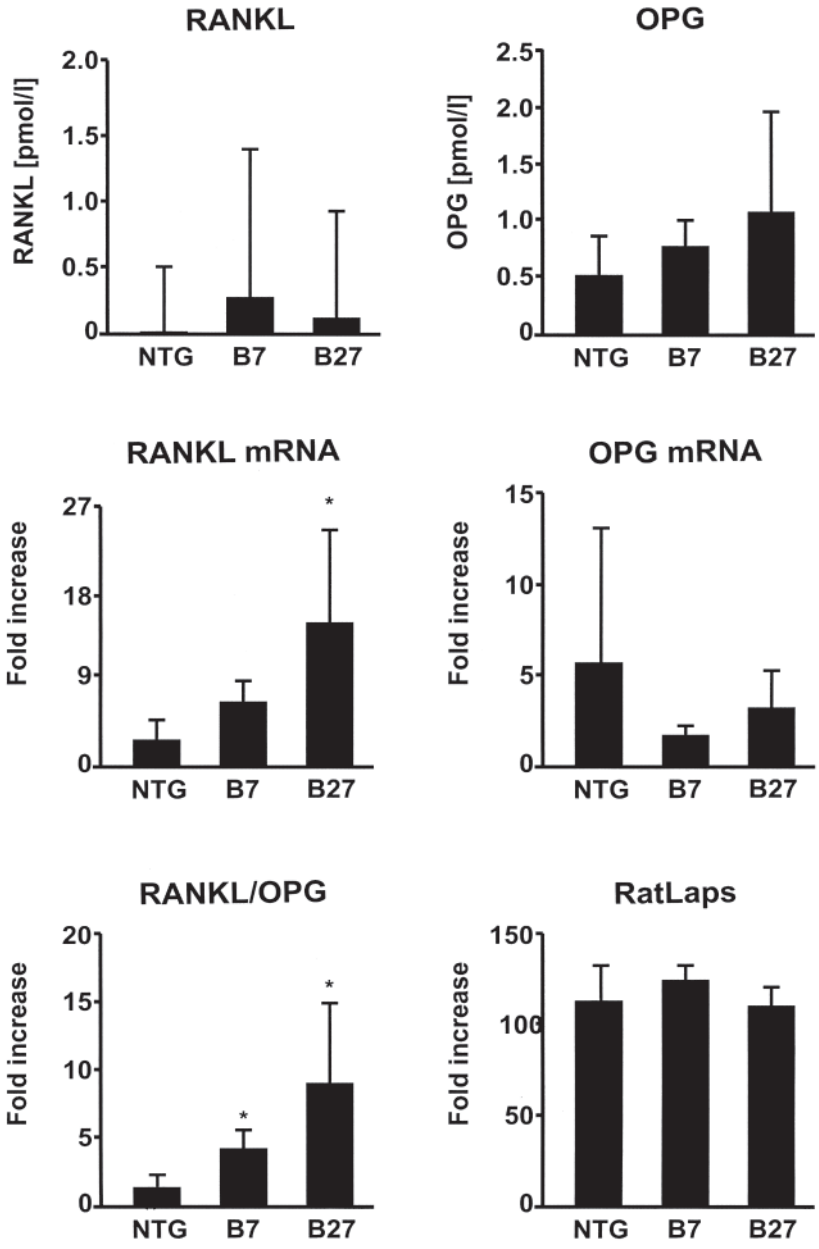

Figure 3. Regulation of the RANKL/OPG system and levels of CTX in HLA-B27 transgenic rats. Serum levels of RANKL, OPG, and CTX (RatLaps) showed no statistically significant difference between groups. RANKL mRNA levels increased significantly in the diseased HLA-B27 transgenic rats compared to the nontransgenic control group (NTG), whereas OPG mRNA levels were not altered significantly. This resulted in an overall increased RANKL/OPG ratio in the HLA-B7 and HLA-B27 transgenic rat groups compared to the NTG group. *$p < 0.05$.

of RANKL and its decoy receptor OPG in the severe bone loss seen in the HLA-B27 transgenic rat model. Studies in patients with SpA showed increased levels of serum RANKL[12]. In that study serum levels of RANKL were mostly below the detection limit, and hence allowed no clear interpretation. With regard to serum OPG concentrations in patients with SpA, research groups have reported contrasting results, showing decreased[10], increased[11], or nearly normal OPG levels[12] compared to healthy controls. In our study, no statistical differences in serum or bone mRNA levels of OPG were found. However, mRNA levels of RANKL and the resulting RANKL/OPG ratio in bone tissue of HLA-B27 transgenic rats were significantly elevated, providing a possible mechanism of the severe bone loss seen in those rats. Interestingly, the RANKL/OPG ratio was also elevated in the HLA-B7 transgenic rats. One possible interpretation of this is that the

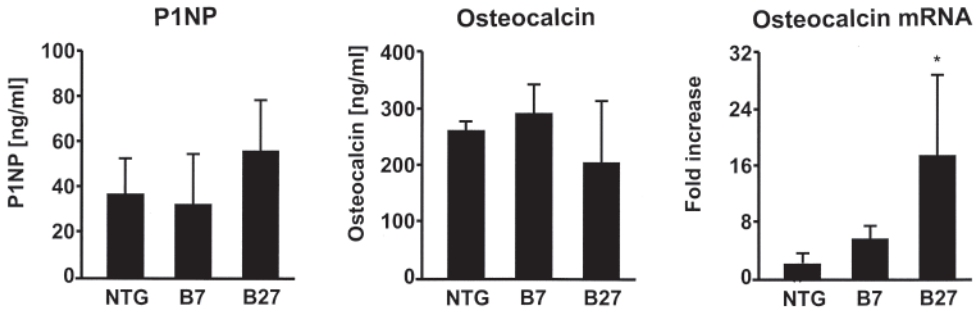

Figure 4. Markers of bone formation in HLA-B27 transgenic rats. Serum levels of N-terminal propeptide of type I procollagen (P1NP) and osteocalcin were not significantly changed in HLA-B27 transgenic rats compared to the nontransgenic (NTG) and HLA-B7 transgenic controls. Osteocalcin mRNA levels were increased in the HLA-B7 and HLA-B27 transgenic rats in the bone tissue compared to the NTG group. *$p < 0.05$.

transgene effect is just more pronounced with HLA-B27, leading to the development of bone loss. Intermediate changes have already been described in HLA-B7 transgenic rats in other circumstances, such as a functional defect of dendritic cells[24]. Possibly, molecules other than RANKL are involved in the pathogenesis of bone loss in HLA-B27 transgenic rats.

Despite the striking bone mass reduction of roughly 40% in the diseased rats, levels of CTX were not increased in the transgenic rats. Since the rats used in our study were an average age of 9 months and had developed disease for several months, it is possible that the process of active bone destruction was already completed and had converted to a bone formation course. Such a sequential process would somehow mimic the disease course of SpA in humans, where osteoporosis usually occurs shortly after disease onset, but the development of bony outgrowths, termed syndesmophytes, increases with disease duration[22]. Supporting this hypothesis, serum levels of P1NP tended to be elevated in the HLA-B27 transgenic rats, and mRNA levels of osteocalcin in bone were significantly increased This may indicate that local matrix mineralization by osteoblasts has already been initiated, whereas systemically, levels of the early osteoblast marker P1NP have just begun to increase, demonstrating the activation of bone formation. This would also explain the not yet increased serum levels of the late osteoblastic marker osteocalcin.

Together, our data indicate that HLA-B27 transgenic rats faithfully mimic bone loss occurring at the early stages of human SpA. Our study suggests that the locally increased RANKL to OPG ratio may contribute to the bone loss in those rats. In future investigations it would be interesting to longitudinally assess the development of bone metabolism in HLA-B27 transgenic rats from disease onset to advanced stages in order to define the sequence of changes. Finally, our observations of increased osteocalcin mRNA expression in the bone tissue of HLA-B27 transgenic rats and reports on osteophyte formation lead to the conclusion that this animal model might also be suitable to investigate bone formation processes that display well reported features and severe complications in human patients with SpA.

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