Osteoblast Function Is Compromised at Sites of Focal Bone Erosion in Inflammatory Arthritis

Nicole C. Walsh,1 Susan Reinwald,2 Catherine A. Manning,1 Keith W. Condon,2 Ken Iwata,2 David B. Burr,2 and Ellen M. Gravallese1

ABSTRACT: In rheumatoid arthritis (RA), synovial inflammation results in focal erosion of articular bone. Despite treatment attenuating inflammation, repair of erosions with adequate formation of new bone is uncommon in RA, suggesting that bone formation may be compromised at these sites. Dynamic bone histomorphometry was used in a murine model of RA to determine the impact of inflammation on osteoblast function within eroded arthritic bone. Bone formation rates at bone surfaces adjacent to inflammation were similar to those observed in nonarthritic bone; therefore, osteoblast activity is unlikely to compensate for the increased bone resorption at these sites. Within arthritic bone, the extent of actively mineralizing surface was reduced at bone surfaces adjacent to inflammation compared with bone surfaces adjacent to normal marrow. Consistent with the reduction in mineralized bone formation, there was a notable paucity of cells expressing the mid- to late stage osteoblast lineage marker alkaline phosphatase, despite a clear presence of cells expressing the early osteoblast lineage marker Runx2. In addition, several members of the Dickkopf and secreted Frizzled-related protein families of Wnt signaling antagonists were upregulated in arthritic synovial tissues, suggesting that inhibition of Wnt signaling could be one mechanism contributing to impaired osteoblast function within arthritic bone. Together, these data indicate that the presence of inflammation within arthritic bone impairs osteoblast capacity to form adequate mineralized bone, thus contributing to the net loss of bone and failure of bone repair at sites of focal bone erosion in RA.

J Bone Miner Res 2009;24:1572–1585. Published online on March 30, 2009; doi: 10.1359/JBMR.090320

Key words: arthritis, inflammation, osteoblast, bone histomorphometry, Wnt

INTRODUCTION

RHEUMATOID ARTHRITIS (RA) is a chronic inflammatory disease characterized by inflammation and hyperplasia of the joint lining (synovium) that results in the destruction of articular cartilage and bone. Focal articular bone loss (or erosion) is mediated by osteoclasts,(1–4) occurs early in RA, progresses rapidly in the absence of treatment, and contributes to joint deformity and patient morbidity.(5)

In RA, the balance between bone resorption and bone formation is perturbed, resulting in net bone loss. Current disease-modifying agents available for the treatment of RA have proven effective in the suppression of inflammation, leading to a slowing or arresting of the progression of focal articular erosions.(6) Although uncommon, repair of focal bone erosions through the formation of new bone has been documented,(7–13) and its occurrence has been associated with the control of inflammation.(14,15)

Osteoblast lineage cells have been identified within the arthritic bone microenvironment. Cells with the morphologic appearance of osteoblasts, expressing PTH receptor (PTHR) mRNA (expressed by postproliferative osteoblast lineage cells), were identified on bone surfaces adjacent to sites of focal bone erosion in human RA.(16) In mice that constitutively express human TNF (hTNF.Tg mice),(17) PTHR-positive cells were evident on endosteal bone surfaces within the vicinity of inflammation.(18) Osteoblast lineage cells expressing mRNA for the bone matrix protein osteocalcin, a marker of mature osteoblasts, were also observed on endosteal bone surfaces adjacent to inflammation.(19) However, fewer osteocalcin-positive cells were evident on bone surfaces adjacent to sites of focal bone erosion in this model.(19) Interestingly, deposition of osteoid (unmineralized bone matrix) was associated with the presence of these osteocalcin-expressing cells on the endosteal bone surfaces adjacent to inflammation.(19,20) Similar sites of osteoid deposition were also documented in human RA.(19) Although the presence of osteoblast-lineage cells and the focal deposition of osteoid have been shown within the RA bone microenvironment, the impact...
of inflammation on the maturation and function of these osteoblasts has not been elucidated.

Osteoblast differentiation and function requires the activation of the canonical Wnt signaling pathway. The interaction of specific Wnt ligands with their respective co-receptors results in the stabilization of cytoplasmic β-catenin, which translocates to the nucleus where it can participate in initiation of transcription of target genes,\(^3\) including the essential osteoblast transcription factor Runx2.\(^{21}\) Inhibition of Wnt signaling by endogenous Wnt signaling antagonists, including members of the Dickkopf (DKK) and secreted frizzled-related proteins (sFRP) families, has been implicated in mediating inhibition of bone formation in settings of pathologic bone remodeling, such as that evident in multiple myeloma\(^{22–26}\) and glucocorticoid-induced osteoporosis.\(^{27–29}\)

It was recently shown that the Wnt signaling antagonist DKK1 contributes to focal bone erosion in RA. Elevated levels of DKK1 were shown in sera from RA patients, and antibody blockade of DKK1 in mouse models of inflammatory arthritis from the time of disease onset resulted in protection from focal bone erosion.\(^{30}\) However, the effects of enhanced Wnt signaling through the blockade of DKK1 on the differentiation state and capacity of osteoblast-lineage cells to form mineralized bone matrix have not been delineated.

In this study, we tested the hypothesis that inflammation at sites of focal bone erosion within arthritic joints not only promotes bone resorption but also inhibits bone formation, contributing to a net loss of bone at these sites. We used the serum transfer model of RA\(^{31,32}\) to determine the capacity of osteoblasts to form mineralized bone at sites of inflammation and bone erosion and to investigate the expression of antagonists of the Wnt signaling pathway within the arthritic joint. Identification of mechanisms that regulate osteoblast function within focal bone erosions in RA is critical for the identification of potential targets for therapeutic intervention leading to augmentation of bone at these sites.

**MATERIALS AND METHODS**

**K/B×N model of serum transfer arthritis**

Ethical approval was obtained from the appropriate IACUC committees for all animal work. KRN-T cell transgenic mice were obtained from Drs. Benoist and Mathis (Joslin Diabetes Center, Boston, MA, USA) and The Institut de Genetique et de Biologie Moleculaire et Cellulaire, (Strasbourg, France). C57BL/6j and NODshii/LtJ mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and were used for breeding of K/B×N arthritic mice. Arthritogenic serum was collected from arthritic K/B×N mice and pooled for use in serum transfer arthritis (STA) experiments as described previously.\(^{3,31,32}\)

STA was generated in 12-wk-old male C57BL/6j mice by intraperitoneal injection of 150 µl of arthritogenic serum on days 0, 2, 7, and 14, with nonarthritic control mice receiving PBS (150 µl). Twelve-week-old mice are beyond the phase of juvenile growth, and the use of males avoids possible confounding hormonal effects.

**Dynamic bone histomorphometry**

To assess osteoblast function in vivo, STA was induced in 15 mice, and 10 mice were used as nonarthritic controls. All mice were administered the fluorochromes alizarin (50 mg/kg) at day 5 after initial serum injection and calcine (30 mg/kg) at day 15 by intraperitoneal injection. Mice were killed on day 18, and the right hind limbs were collected, fixed for 3 days in 10% neutral buffered formalin, and embedded in methylmethacrylate for assessment of bone formation activity.

In STA, the navicular bone within the hind paw is a reproducible site of focal bone erosion and was therefore chosen for histomorphometric analysis. In arthritic mice, bone surfaces adjacent to normal marrow at sites where inflammation had not yet penetrated, and bone surfaces adjacent to pannus and inflammation were quantitated separately. In a subset of these mice (seven arthritic, eight nonarthritic), the distal femur, a site remote from inflammation, was analyzed for bone formation parameters to determine the specificity of findings at sites of focal bone erosion. A subset of samples was used for these analyses, because there was little variability among samples in this region. Measurements were taken at a site located 0.8 mm from the growth plate and encompassing 7–8 mm², with both trabecular and endocortical surfaces included in the assessment.

Histomorphometric measurements quantitating alizarin and calcine label incorporation into newly formed bone were performed on three sections (5 µm each, separated by five sections) using a semiautomated analysis system (Bioquant OSTEO 7.20.10; Bioquant Image Analysis) attached to a microscope with both visible and UV light sources (Optiphot 2 microscope; Nikon). Calculations indicative of bone formation were as described by Parfitt et al.\(^{33}\) For calculation of mineralized surface/bone surface (MS/BS, %), the values for the single red and green labels were combined into one measure. The equation used for determining MS/BS also accounts for label escape: MS/BS = (dL + (0.5 × sL))/(sL + dL + nL) × 100. Mineral apposition rate (MAR, µm/d), was calculated by determining the interlabel widths (Ir.Wi) between the alizarin and calcine labels from all three sections and subsequently averaged and divided by the 10-day interlabel time period (average Ir.Wi/10). The bone formation rate/bone surface (BFR/BS, µm³/µm²/yr) was calculated as BFR/BS (µm³/µm²/yr) = MS/BS × MAR × 365. When double labeling was absent in a sample, but single label was present, the MAR value used for calculation of BFR/BS was 0.3.\(^{34}\)

For further histologic analysis of undecalcified hind paws, sections adjacent to those analyzed for dynamic bone histomorphometry were stained with Toluidine blue or Goldner’s trichrome stains.\(^{35}\) The presence of nonmineralized matrix (osteoid) within the navicular bone was assessed by quantitation of osteoid staining present in Goldner’s-stained sections. Three sections per sample (five sections apart) were assessed, and measurements were summed to obtain a single measure per sample. The
percentage of bone surface covered by osteoid per total bone surface (O.Pm/T.Pm) was calculated. For identification of osteoclasts, thin sections were deplasticized in acetone and stained for TRACP.(36)

**Histologic and gene and protein expression analyses**

To assess histologic changes across the time course of arthritis and associate these with the presence and differentiation state of osteoblast lineage cells and with expression of genes of interest, tissues were collected from 18 mice killed throughout the course of STA. Mice were killed as follows: day 7, five mice; day 8, two mice; day 10, three mice; day 12, two mice; day 15, four mice; day 18, two mice. Tissues from four nonarthritic mice were used as controls. For histologic, immunohistochemical, and in situ hybridization studies, left hind limbs were fixed overnight in 4% paraformaldehyde and then placed in decalcification solution (15% EDTA in PBS/0.5% PFA) for a maximum of 2 wk. Intact hind paws were embedded in paraffin, and 5-mm sections were cut for analyses. For analysis of gene expression by RT-PCR, right hind limbs were placed in RNA-later (Ambion) and stored for RNA isolation and gene expression analyses.

**Immunohistochemistry**

Immunohistochemical staining using serial sections from arthritic and nonarthritic hind paws was performed to identify osteoblast lineage cells expressing protein for markers specific to the stages of osteoblast differentiation including Runx2 (early osteoblast), alkaline phosphatase (mid- to late-stage osteoblast), and osteocalcin (late-stage osteoblast). Immunohistochemistry was performed as described previously.(37) Paraffin sections of hind paws (including mid-distal tibia) were dewaxed in xylene and rehydrated through a decreasing gradient of ethanol. For antigens requiring microwave retrieval, slides were heated to 93°C before cooling for a minimum of 2 h at room temperature. Antibodies and conditions used for antigen retrieval are provided in Table 1. Bound antibodies were visualized using DAB liquid chromogen (DAKO), and sections were counterstained using Mayer’s hematoxylin and mounted in Cytoseal 60 (Richard Allan Scientific). Positive antibody staining was identified by the presence of brown color.

**In situ hybridization**

PCR products specific for alkaline phosphatase and osteocalcin were cloned into the pGEMT-easy vector (Promega; refer to Table 2 for primer sequences). Linearized plasmids were used as templates for sense and antisense riboprobe synthesis using T7 and SP6 RNA polymerases (Promega) and DIG-UTP RNA labeling nucleotide mix (Roche) according to the manufacturer’s instructions. In situ hybridization was performed on tissue sections from arthritic hind paws according to previously described methods.(38,39) Bound riboprobes were detected by incubating sections in NBT/BCIP in 100 mM Tris-HCl, 100 mM NaCl, and 50 mM MgCl₂, pH 9.5. Staining was allowed to proceed for 16–18 h, and slides were mounted in Aquamount (VectaLabs).
Brightfield microscopy

A transmitted light microscope (Nikon, Tokyo, Japan) and AxioCam digital camera (Carl Zeiss International) run by Openlab imaging software was used for image capture and analysis.

Analysis of gene expression in synovial and associated soft tissues

Total RNA was isolated from tissue samples using a modified acid-guanidinium isothiocyanate phenol-chloroform extraction procedure. Synovial and associated soft tissues from the tibio-talar joint were macrodissected from arthritic and nonarthritic hind paws collected across the time course of STA. Tissues were ground to powder in the presence of liquid nitrogen before the addition of Solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl) and subsequent isolation of total RNA. RNA samples were treated with DNA-free (Ambion to remove contaminating genomic DNA.

cDNA was synthesized from 1 µg RNAs obtained from each synovial soft tissue sample using the IScript cDNA synthesis kit (BioRad). Preliminary gene expression studies using semiquantitative RT-PCR were conducted using a small sample of available RNAs (data not shown). qRT-PCR for mRNA expression was performed using the remaining RNA samples using IScript Sybr Green RT-PCR mix (BioRad) and a Realplex qPCR machine (Eppendorf). Sample numbers were as follows: nonarthritic controls and arthritic, days 8, 15, and 18, two mice per time point; arthritic days 10 and 12; one mouse per time point. All PCRs were run in triplicate. Primers for hydroxymethylbilane synthase (HMBS, housekeeping gene), sFRP1, sFRP4, and DKK3 were as described in Table 2. Quantitech Sybr Green primers (Qiagen) were purchased for amplification of DKK1, DKK2, and sFRP2. Expression of the gene of interest was normalized to expression of HMBS and fold-change for arthritic:nonarthritic and fold-change for arthritic:nonarthritic

Statistical analysis

Microsoft Excel was used for data collation and calculation of bone histomorphometric measurements. Prism 5.0 (GraphPad) was used for graphing and statistical analysis. Student’s t-test was used to analyze significance for data with normal distribution; an unpaired t-test was applied to analyses between arthritic and nonarthritic samples; and a paired t-test was applied to analyses between bone surfaces adjacent to inflammation and bone surfaces adjacent to normal marrow within arthritic bone. The Mann-Whitney U-test was applied to variables that did not show normal distribution.

RESULTS

Focal erosion of bone associated with invasion of inflamed synovial tissue occurred rapidly within the hind paws in the STA murine model. Histologic analysis of tissue sections collected across a time course of arthritis identified the navicular bone as a reproducible site of erosion in this model, with osteoclast-mediated bone destruction evident from day 5 after initial serum injection and full-thickness cortical erosion present from approximately day 10 onward (data not shown). The navicular bone was therefore chosen as a site to evaluate the development and progression of erosion and the contribution of osteoblasts to the process of articular erosions.

Defined pathologic changes occurred within arthritic bone as inflamed synovially derived tissue invaded into the cortical bone and bone marrow spaces. Histologic observation identified three distinct compartments within the arthritic navicular bone (Fig. 1A). The first consisted of bone surfaces adjacent to invading synovial tissue (pannus) and inflamed synovium. The second consisted of bone surfaces within the eroded bone that were distal to the invading inflammatory tissue and adjacent to normal bone marrow, as identified by the presence of normal marrow cellularity and interspersed fat and the absence of inflammatory tissue. These two regions were analyzed to determine the function of osteoblast lineage cells within arthritic bone. The third compartment was comprised of periosteal bone surfaces with adjacent inflammation. In STA, as in the collagen-induced arthritis and antigen-induced arthritis rodent models, there is a unique response to the presence of inflammation at this site, with the initiation of new bone formation that resembles osteophytes. Osteoclasts, identified by staining for TRACP activity, are clearly evident at bone surfaces adjacent to inflammation in arthritic navicular bone and are rare in nonarthritic bone (Fig. 1B).

Formation of mineralized bone in inflammatory arthritis is reduced at bone surfaces directly adjacent to inflammation compared with bone surfaces adjacent to normal marrow

Newly formed mineralized bone was labeled in vivo by the incorporation of the fluorochromes, alizarin (fluoresces red) and calcine (fluoresces green) (Figs. 1C and 1D).
Assessment of unstained sections of nondecalcified arthritic hind paws showed that formation of mineralized bone did occur both at bone surfaces adjacent to inflammation (Fig. 1C, triangles) and at bone surfaces adjacent to normal marrow (squares). Arrows identify osteophyte formation on periosseal bone surface, which is often observed in animal models of inflammatory arthritis. Scale bar, 100 μm. (B) TRACP-stained tissue sections from arthritic and nonarthritic bone identifying osteoclasts (red staining, arrows) at bone surfaces adjacent to inflammation (triangles). Scale bar, 100 μm. (C) Unstained sections showing alizarin (red) and calcine (green) incorporation identifying mineralized bone within arthritic navicular bone. Photographs shown in (i) and (ii) are from two different mice [original magnification: (i) ×100 and (ii) ×200]. (D) Representative photomicrograph of alizarin and calcine labeling in nonarthritic navicular bone showing clear incorporation of bone fluorochrome labels (original magnification: ×200). (E and F) Quantitation of bone histomorphometric parameters within arthritic and nonarthritic navicular bones. (E) Bone formation rate/total bone surface (BFR/BS). (F) Mineralized surface/total bone surface (MS/BS). Each symbol represents measurement from one animal. Horizontal line indicates the mean value for each group measurement. Statistical significance determined by Student’s t-test.

Assessment of unstained sections of nondecalcified arthritic hind paws showed that formation of mineralized bone did occur both at bone surfaces adjacent to inflammation (Fig. 1C, triangles) and at bone surfaces juxtaposed to normal marrow (Fig. 1C, squares). Alizarin and calcine label incorporation was also observed at bone surfaces within nonarthritic bone (Fig. 1D).

No difference was observed in bone formation rate/total bone surface (BFR/BS, %), between bone surfaces adjacent to inflammation within arthritic bone and nonarthritic bone surfaces (Fig. 1E). At bone surfaces adjacent to inflammation (triangles), less fluorochrome incorporation was evident compared with bone surfaces adjacent to normal marrow (squares). This was clearly shown on
determination of mineralized surface per total bone surface (MS/BS, %) at these distinct sites (Fig. 1F). MS/BS (%) was reduced by $35\% (p = 0.011)$ at bone surfaces adjacent to inflammation compared with bone surfaces adjacent to normal marrow within the arthritic navicular bone. Consistent with this observation, the calculated bone formation rate per total bone surface (BFR/BS, $\mu m^3/\mu m^2/yr$) was $\sim40\%$ lower at sites of inflammation ($p = 0.013$; Fig. 1E).

Alterations in osteoblast function within arthritic bone are a result of the local presence of inflammation

At the distal femur, a site remote from inflammation in STA, no significant difference was observed between arthritic and nonarthritic mice in MS/BS, MAR, or BFR/BS (Fig. 2), showing that the effects observed within the arthritic navicular bone were a result of local inflammation and not a reflection of a systemic effect of inflammation on bone remodeling.

On bone surfaces within arthritic bone, there is an abundance of osteoblast-lineage cells expressing Runx2 but a more limited number of cells expressing alkaline phosphatase and osteocalcin. Immunohistochemical staining of tissue sections from arthritic hind paws showed that osteoblast lineage cells expressing Runx2 protein were abundant at bone surfaces adjacent to inflammation in arthritic bone (Fig. 3Aii, solid arrows). Analysis of serial sections showed that these Runx2-expressing cells were mostly negative for alkaline phosphatase and osteocalcin proteins (Figs. 3Aiii and 3Aiv), indicating that these cells were likely to be osteoblast progenitor cells. In contrast, cells lining trabecular bone within the distal tibia, a site remote from focal bone erosion, expressed both alkaline phosphatase and osteocalcin proteins (Fig. 3B, open arrows).

In situ hybridization for alkaline phosphatase mRNA and osteocalcin mRNA on serial sections was also performed. This confirmed that, within arthritic bone, despite the abundance of osteoblast lineage cells expressing Runx2 protein (Fig. 3C, solid arrows), few cells weakly expressing osteocalcin mRNA were present at the inflammation–bone interface (Fig. 3D, solid arrow). Limited expression of osteocalcin mRNA was also evident in cuboidal-shaped mesenchymal cells adjacent to inflammation within the arthritic bone, whereas osteocalcin expression was high in trabecular bone lining normal marrow without inflammation within the distal tibia (Fig. 3E, open arrows).
osteoblast-lineage cells located at bone surfaces adjacent to normal marrow (Fig. 3D, open arrow). Similar to the pattern of alkaline phosphatase protein expression, alkaline phosphatase mRNA expression was minimal to absent within the arthritic bones, both at sites of inflammation and on bone surfaces adjacent to normal marrow (Fig. 3Dii). In contrast, cells strongly expressing osteocalcin and alkaline phosphatase mRNAs were present at sites of periosteal new bone formation on the navicular bone (Fig. 3E, red arrows) and on trabecular surfaces within the distal tibia (data not shown). This pattern of expression was consistently observed in samples obtained from the onset of established erosion at day 7 until death at day 18. Similar observations were made in histologic sections of arthritic joints obtained from mice induced with AIA, a T cell–driven arthritis model of RA (data not shown).

Mineralization of bone is impaired within arthritic bone

The MAR (μm/d), indicative of the individual activity of osteoblasts, was increased ~2-fold at bone surfaces adjacent to normal marrow within arthritic bone compared with bone surfaces within nonarthritic bone (p = 0.006; Fig. 4). Despite this increase in capacity to form bone, there was no difference in the extent of the actively mineralized surface as measured by MS/BS (%) at these sites compared with nonarthritic bone (Fig. 1F). Furthermore, within arthritic bone, sites adjacent to inflammation and normal marrow did not show a significant difference in MAR (p = 0.159), indicating similar bone formation activity of individual osteoblasts (Fig. 4), but MS/BS was reduced at bone surfaces adjacent to inflammation (Fig. 1F).

Bone surfaces adjacent to inflammatory tissue, and to a lesser extent normal marrow, had a substantial layer of bone matrix overlying the mineralized bone surfaces. Serial sections stained with toluidine blue (identifying proteoglycan content), and Goldner’s stain (which distinguishes nonmineralized bone [osteoid, pink] from mineralized bone tissues [green]) indicated that this tissue was different in appearance to nonarthritic bone and suggested that the bone matrix in these areas was only partially mineralized (indicated by partial light-green staining; Figs. 5A–5C).

The extent of osteoid-covered surfaces (osteoid perimeter/total perimeter [O.Pm/T.Pm, %]) was quantitated in all arthritic samples. Five arthritic mice had only bone surfaces adjacent to inflammation. Ten arthritic mice had bone surfaces adjacent to normal marrow and bone surfaces adjacent to inflammation. Although there was no difference in O.Pm/T.Pm when animals were assessed as groups, 7 of 10 samples that had both histologic regions showed increased O.Pm/T.Pm (%) at bone surfaces adjacent to inflammation compared with bone surfaces adjacent to normal marrow (Fig. 5D).

**Wnt signaling antagonists of the DKK and sFRP families are expressed by cells within invading synovial tissues in inflammatory arthritis**

qRT-PCR showed that DKK1 mRNA expression was elevated in arthritic synovial tissues above the expression levels in nonarthritic mice over the course of STA, with peak expression (an increase of 5-fold compared with nonarthritic control tissues) occurring around day 12 after initial serum injection, when inflammation and focal bone erosion are typically at peak (Fig. 6A). Unlike DKK1, sFRP1 mRNA was significantly elevated above expression levels in nonarthritic mice from the early stages of arthritis (Fig. 6A), corresponding to the progression of inflammation and the onset of focal bone erosion within nearby bones. Immunohistochemical analysis confirmed that both DKK1 and sFRP1 proteins were expressed by cells predominantly located within the inflamed synovial tissues and associated soft tissues across the time course of arthritis (Fig. 6B, open arrows). Some bone-lining cells (Fig. 6B, solid arrows) and occasional osteoclasts (data not shown) were also observed to express both factors. Low-level expression in the nonarthritic hind paws was limited to cells present within the synovial lining layer (data not shown).

qRT-PCR screening for expression of other members of the DKK and sFRP families of Wnt signaling antagonists showed that DKK2, DKK3, sFRP2, and sFRP4 mRNA expression levels within arthritic synovial tissue and associated soft tissues were also elevated compared with nonarthritic tissues (Fig. 7). Interestingly, expression of mRNA for DKK2, DKK3, and sFRP4 mRNAs was highest compared with nonarthritic mice noted at late stages of disease. Similar to sFRP1 mRNA, sFRP2 mRNA was highly expressed from early stage of disease.

**DISCUSSION**

Pathologic bone remodeling is often characterized by uncoupling of bone formation and bone resorption, leading to a perturbation of skeletal integrity. In multiple myeloma, for example, increased osteoclastic resorption(25,45,46) is accompanied by a suppression in the capacity of the
Osteoblast to form bone. In RA and in animal models of inflammatory arthritis, increased osteoclast differentiation and subsequent bone resorption occurs at the interface of inflammatory tissue and bone, resulting in significant bone loss and compromised skeletal integrity. In addition, repair of bone erosion is uncommon, suggesting that, as in myeloma, bone formation may also be compromised in this disease, augmenting bone loss at these sites. Using the approach of dynamic bone histomorphometry, we showed that the presence of inflammation within arthritic bone impairs osteoblast capacity to form adequate mineralized bone, thus contributing to the net loss of bone and failure of bone repair at sites of focal bone erosion in RA.

If bone formation was coupled to bone resorption at sites of focal bone erosion in RA, we would predict that bone formation would be elevated at these sites compared with nonarthritic bone in which the rate of bone turnover is limited. In this study, the formation of mineralized bone was evident at bone surfaces adjacent to inflammation within eroded arthritic bone. However, the rate of bone formation at these sites was similar to that in nonarthritic bone. This suggests that osteoblast activity at sites of focal bone erosion is not likely to compensate for the known osteoclastic resorption at these sites and is consistent with an uncoupling of the activities of these two cell types. Furthermore the rate of bone formation at bone surfaces adjacent to inflammation was reduced compared with bone surfaces adjacent to normal bone marrow within arthritic bone, suggesting that the presence of inflammatory tissue adjacent to bone directly impacts osteoblast bone formation activity.

FIG. 5. Accumulation of partially mineralized bone matrix is evident on bone surfaces adjacent to inflammation. Serial sections of nondecalcified hind paws showing the navicular bone from arthritic and nonarthritic mice killed 18 days after initial serum injection. Representative images are shown for arthritic bone surfaces adjacent to (A) inflammation (triangles), (B) normal bone marrow (squares), and (C) bone surfaces within nonarthritic bone. Unstained sections show alizarin (red) and calcine (green) incorporation indicating mineralized bone surfaces. Toluidine blue staining shows the presence of proteoglycans in bone matrix (dark purple staining) identifying newly formed bone matrix. Goldner’s stain distinguishes between mineralized bone matrix (dark green staining) and unmineralized bone matrix (osteoid, pink staining) and shows partially mineralized bone (light green staining) in arthritic bone adjacent to normal marrow and inflammation (arrows). (D) The osteoid perimeter/total bone perimeter (O.Pm/T.Pm) was measured for bone surfaces in arthritic bone adjacent to normal marrow or inflammation. Five mice had only bone surfaces adjacent to inflammation and these are shown as individual triangles. Line plot show results for the 10 arthritic mice that had bone surfaces adjacent to inflammation (triangles) and adjacent to normal bone marrow (squares). Each individual line represents measurements from one animal for osteoid at these bone surfaces to show the relationship between osteoid deposition at these sites.
Dynamic bone histomorphometry quantifies the initial phase of mineralization of the newly deposited bone matrix. Our data also indicate that, within arthritic bone, this primary phase of bone mineralization is impaired by the presence of inflammatory tissue. Although individual osteoblasts at bone surfaces adjacent to inflammation had a similar capacity to form bone matrix compared with osteoblasts located adjacent to normal bone marrow (indicated by similar MARs), the extent of actively mineralizing surface (quantitated by MS/BS, indicative of the combined production of mineralized bone by osteoblast lineage cells) is significantly reduced at bone surfaces adjacent to inflammatory tissue compared with bone surfaces adjacent to normal marrow. The increase in osteoid-covered surface (quantitated by O.Pm/T.Pm) at bone surfaces adjacent to inflammation compared with bone surfaces adjacent to normal marrow in the 7 of 10 mice that had both bone microenvironments, and the presence of a partially mineralized layer of bone matrix overlying these bone surfaces, are also consistent with the decrease in mineralization activity measured at bone surfaces adjacent to inflammation.

It is possible that the increase in bone remodeling activity at bone surfaces adjacent to inflammation could contribute to reduction in the extent of actively mineralizing surface at these sites. Similar to the effect of increased bone turnover on mineralization of bone in postmenopausal osteoporosis, the increase in osteoclast activity at bone surfaces adjacent to inflammation in RA may simply not allow enough time for efficient mineralization of bone before the next cycle of resorption.

Interestingly, distal effects of invading inflammatory tissue on osteoblast activity at bone surfaces adjacent to normal bone marrow within arthritic bone were also evident. A 2-fold increase in bone formation capacity (shown by MAR and BFR/BS) was observed at these surfaces compared with nonarthritic bone. However, the extent of actively mineralizing surface (indicated by MS/BS) at these surfaces was similar to nonarthritic bone. Furthermore a layer of partially mineralized bone matrix was consistently observed overlying these bone surfaces but to a lesser extent than bone surfaces directly adjacent to inflammation. Together these observations indicate that an imbalance...
between bone formation capacity and extent of mineralization activity also exists at these sites and may be analogous to the demineralization of bone that is often observed in RA patients at periarticular sites where bone turnover is increased (sites juxtaposed to, but not directly adjacent to, invading inflammatory tissue).  

The abundance of cells expressing the early osteoblast lineage marker Runx2 and the presence of fewer cells expressing alkaline phosphatase and the mature osteoblast marker osteocalcin suggests that osteoblast maturation to a fully functional cell is impaired by the presence of invading inflammatory tissue. This supports the concept that there are fewer sites at which mature, fully functional osteoblast lineage cells are present and/or active at bone surfaces adjacent to inflammation and is consistent with the decrease in the extent of actively mineralizing surface at these sites compared with bone surfaces adjacent to normal bone marrow within arthritic bone.

Notably, the expression of alkaline phosphatase, a mid-to late-stage osteoblast marker that is required for efficient mineralization of bone matrix, is markedly limited within sites of eroded bone, despite intense expression of this enzyme at sites of new bone formation located on the periosteal bone surface. The deficiency of alkaline phosphatase expression by osteoblast lineage cells within the arthritic bone may contribute to the defect in the capacity of these cells to mineralize bone matrix and warrants further study.

It is difficult to determine whether the effect of inflammation on osteoblast maturation is gene specific, that is, restricted to the downregulation of gene expression for alkaline phosphatase and to a lesser extent, osteocalcin, or if it is a direct effect on osteoblast differentiation per se. Interestingly, a prior study also showed a paucity of mature osteoblast lineage cells expressing osteocalcin mRNA within arthritic navicular bones obtained from rats with AIA. No other study has examined the extent of alkaline phosphatase expression within the arthritic bone microenvironment.

In physiologic bone remodeling, it is the early to mid-stage osteoblast lineage cells that are primarily responsible for production of the key osteoblast differentiation factor RANKL. RANKL production by osteoblast lineage cells expressing PTHR has been identified on endosteal bone surfaces within the vicinity of bone marrow infiltrates in the hTNF.Tg mouse. In our own studies, RANKL mRNA expression is increased across the time course of STA, with limited expression of osteoprotegerin, the decoy receptor for RANKL, within inflamed synovial tissues (data not shown). This is consistent with protein localization studies in human RA. It is possible, therefore, that early osteoblast lineage cells present within arthritic bone that do not mature properly to cells that form mineralized bone may exacerbate focal bone loss through the production of RANKL, which would act to promote local osteoclast differentiation and function.

Data presented in this study suggest that the presence of inflammation within the arthritic bone microenvironment is associated with impaired osteoblast function. Factors produced by cells within the inflammatory infiltrate are likely candidates for contributing to impaired osteoblast function in this setting. We showed that cells within the inflamed synovial tissues express the Wnt signaling antagonists DKK1 and sFRP1 and that their expression is induced in the setting of inflammatory arthritis. In addition, expression of DKK2, DKK3, sFRP2, and sFRP4 mRNA is also upregulated in arthritic synovial tissue and associated soft tissues. This invites the possibility that several Wnt
signaling antagonists expressed within the arthritic bone microenvironment may contribute to the modulation of Wnt signaling in this setting. Inhibition of the canonical Wnt signaling pathway during the early stages of osteoblast differentiation results in a decrease in alkaline phosphatase expression and activity and a reduction in mineralization capacity.\(^{(63,64)}\) This effect is consistent with the paucity of alkaline phosphatase expression by osteoblast lineage cells and the deficit in mineralized bone formation at bone surfaces adjacent to inflammation within the arthritic bone microenvironment.

The function of DKK1 in the setting of RA was recently elucidated in studies reported by Diarra et al.\(^{(30)}\) Elevated levels of DKK1 were observed in arthritic hTNF.Tg mice and in sera obtained from RA patients. In the hTNF.Tg mouse, prophylactic treatment with an antibody to DKK1 from the onset of inflammatory arthritis resulted in protection from focal bone erosion, with activation of periodontal osteocyte formation otherwise rarely observed in the hTNF.Tg mouse. Protection from erosion was in part caused by the upregulation in osteoprotegerin expression (which binds and inhibits RANKL) and subsequent inhibition of osteoclastogenesis.\(^{(30,65)}\) Importantly, in support of the inhibition of bone formation by DKK1 in animal models of RA, increased deposition of osteoid and bone formation rates within tarsal bones of the affected hind paws was observed with DKK1 inhibition.\(^{(30)}\) However, the effect of Wnt signaling activation through DKK1 blockade on osteoblast differentiation, and the capacity of these cells to form mineralized bone within the arthritic bone microenvironments, was not assessed.

Additional factors produced by cells present within the RA inflammatory tissues, such as the proinflammatory cytokine TNF, are also likely to contribute to inhibition of osteoblast maturation and function at sites of focal bone erosion. TNF has been shown in vitro to act directly on osteoblast lineage cells to downregulate alkaline phosphatase, type 1 collagen, and osteocalcin mRNA expression, resulting in decreased formation of mineralized bone.\(^{(66–71)}\) TNF could also elicit its effect on osteoblast function within the RA bone microenvironment indirectly through modulation of Wnt signaling through the induction of DKK1 in synovial fibroblast cells.\(^{(30)}\) In the STA model, TNF expression is upregulated across the time course of arthritis (data not shown) and is therefore a candidate for modulating osteoblast function in this setting. However, this model also has significant induction of IL-1 expression across the course of disease (data not shown), and the role of IL-1 in specifically modulating osteoblast function within the arthritic bone microenvironment is yet to be determined.

Further signals inhibitory to osteoblast maturation and function are likely to stem from the local microenvironment created by the presence of inflammatory tissue. The mass of metabolically active cells present within the arthritic joint contribute to a hypoxic microenvironment in which there is low oxygen tension,\(^{(72,73)}\) accompanied by increased acidity. Both hypoxia and acidic conditions have been shown to downregulate alkaline phosphatase expression in osteoblast lineage cells in vitro with an associated reduction in mineralization capacity.\(^{(74,75)}\) In addition, hypoxic conditions have been shown to modulate Wnt signaling both directly, through modulation of β-catenin transcriptional activity in osteoblast-like cells,\(^{(76)}\) and indirectly, through the upregulation of DKK1, as shown in myeloma cells.\(^{(77)}\)

Thus, in the setting of inflammatory arthritis, the physical characteristics, cellular composition of the invading inflammatory tissue, and the local production of factors by cells within this infiltrate are likely not only to regulate osteoclast differentiation and focal bone erosion, but also to directly or indirectly modulate osteoblast function leading to net bone loss. It is intriguing that, in STA and other models of inflammatory arthritis, the presence of this same inflammatory tissue on the adjacent periosteal surface of affected bones results in the apposition of new bone in the form of osteophytes.\(^{(42–44)}\) This highlights that the effect of inflammation on bone surfaces within arthritic bone is specific to the immediate microenvironment and underscores the difficulties in translating in vivo observations to in vitro culture systems for the elucidation of mechanisms associated with focal bone loss.

Together our data show that, within the arthritic bone microenvironment, the local presence of inflammation impairs the maturation of cells to fully functional osteoblasts capable of efficient mineralization of bone. This impaired function of osteoblasts is likely to contribute to net loss of bone and failure of bone repair at focal bone erosion sites in RA. The exact role of each of the members of the DKK and sFRP families of Wnt signaling antagonists whose expression is upregulated within the setting of inflammatory arthritis remains to be determined. These factors may act in combination to directly regulate osteoblast and osteoclast differentiation and function and are therefore potential candidate factors for therapeutic targeting. In addition to controlling inflammation, the augmentation of bone formation through modulation of osteoblast function may be a complementary therapeutic strategy for articular bone loss in RA.

ACKNOWLEDGMENTS

This work was funded by Arthritis National Research Foundation and The Sontag Foundation (NCW); Worcester Biomedical Research Foundation (EMG). We thank Jack Ratliff for contributions to a pilot study not reported in this paper.

REFERENCES

OSTEOBLAST FUNCTION IN INFLAMMATORY ARTHRITIS


Received in original form August 12, 2008; revised form February 5, 2009; accepted March 25, 2009.