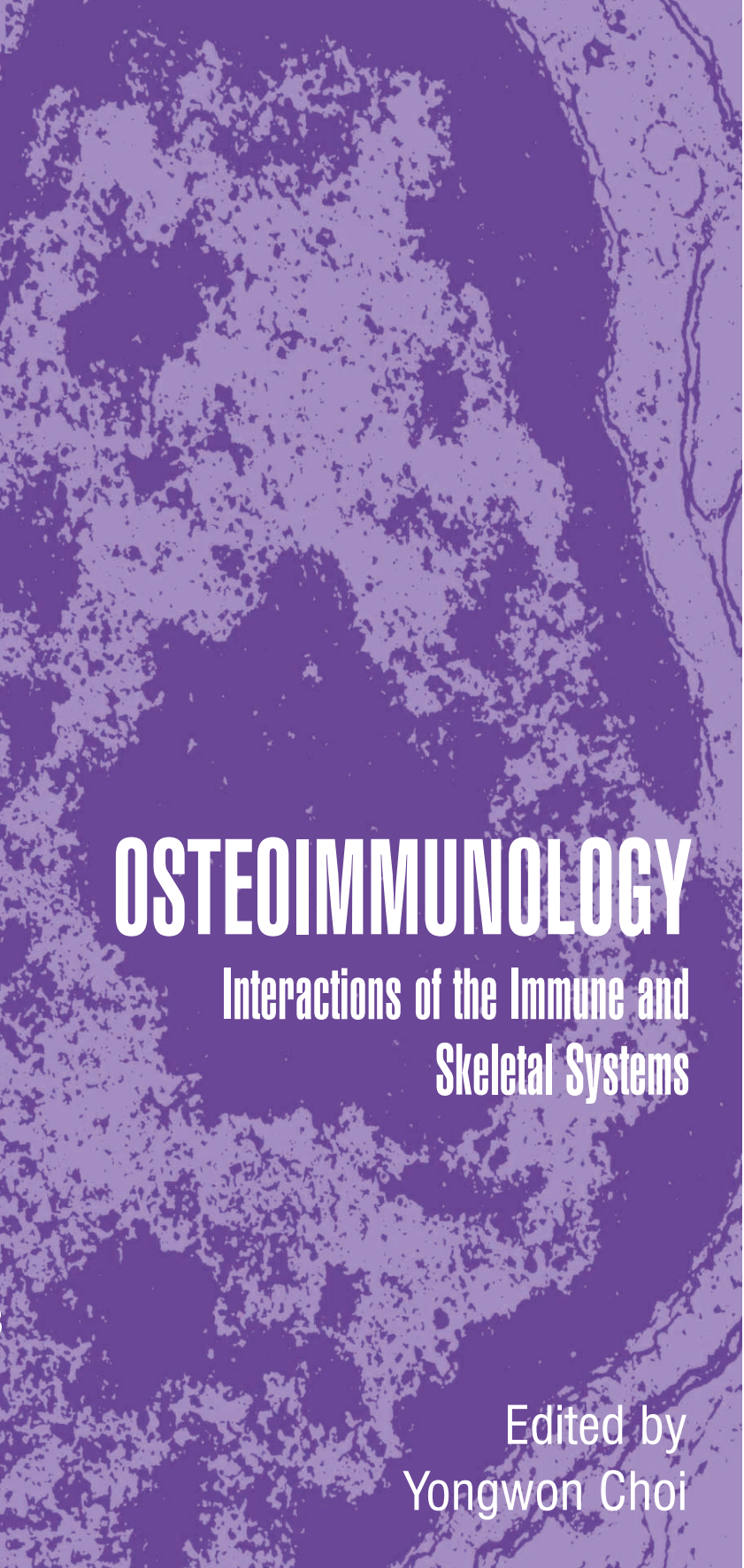

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Volume 602

OSTEOIMMUNOLOGY

Interactions of the Immune and Skeletal Systems

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PREFACE

William J. Sharrock[1]

It's now nine years since the "Workshop on Bone and the Hematopoietic and Immune Systems," sponsored in August 1997 by the NIAMS and several other components of the NIH (Sharrock 1998). Even in 1997, although no one had yet coined the term "osteoimmunology," it was not original to suggest that the skeleton and the immune system might share regulatory pathways or interact with one another directly. The possibility had been raised many times in papers and reviews over a number of years. After all, osteoclasts originate in the monocyte-macrophage lineage, and osteoblasts cohabit with immune cell lineages in the bone marrow. It was also tempting to see significance in such observations as the deleterious effects of immunosuppressants on bone, immune deficiencies in osteopetrosis, or the ubiquity of interleukins and other immune-related cytokines in the pathways that regulate bone remodeling. But suggestions are no substitute for hard evidence, and in 1997 the latter was still in short supply. Reports were scarce, seldom definitive, and often conflicting.

In fact, late 1997 also produced the first outlines of what is arguably the most pivotal junction so far detected in the tangled pathways that regulate the immune and skeletal systems. Choi and colleagues (Wong, Josien, Lee, et al. 1997) described a new member of the TNF receptor family, which they named TRANCE, with significant functions in T lymphocytes and dendritic cells. Almost simultaneously, Anderson et al. (Anderson, Maraskovsky, Billingsley, et al. 1997) described a pair of interacting proteins with effects on T lymphocytes and dendritic cells, naming them RANK and RANK ligand (RANKL). A few months later, Suda and colleagues (Yasuda, Shima, Nakagawa, et al. 1998) reported that the osteoclast differentiation factor (ODF) they had studied for several years was identical to both TRANCE and RANKL. Also in 1998, Lacey et al. (Lacey, Timms, Tan, et al. 1998) identified a ligand for a bone-protective factor called osteoprotegerin (OPG) and labeled it OPGL. In short order, it became clear that OPGL was the same molecule as TRANCE, RANKL, and ODF. Perhaps

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the single most critical regulatory interaction for osteoclastogenesis had been revealed as one with clear functions as well in the immune system, and the literature had incorporated a nomenclatorial knot that surely perplexes graduate students to this day. In 1999, Penninger and colleagues demonstrated the importance of the molecule now most often referred to as RANKL for both the skeleton and the immune system by characterizing mice lacking RANKL (Kong, Yoshida, Sarosi, et al. 1999). These mice are profoundly osteopetrotic, lacking osteoclasts entirely. They also lack lymph nodes and exhibit defects in lymphocyte differentiation. Tellingly, Penninger and colleagues also demonstrated that activated T lymphocytes could induce bone destruction, via RANKL signaling, in a model of inflammatory arthritis (Kong, Feige, Sarosi, et al. 1999).

RANK/RANKL signaling remains a major theme in osteoimmunology in 2006, and is now the target of several innovative therapeutic strategies. In addition, new observations and new investigators have emerged to establish new links between skeletal biology and immunology, and these developments are much in evidence in this volume. Glimcher and colleagues, for example, report on Schnurri-3, a protein that first attracted attention because of its functions in the immune system. Study of mice lacking Schnurri-3, however, reveals the protein's key role in the regulation of postnatal bone mass. Other reports in this volume reflect continuing interest in questions that already figured prominently in the 1997 workshop. For example, Kronenberg and colleagues shed new light on the relationship between osteoblastic and hematopoietic lineages, showing that PTH/PTHrP receptor signaling has important effects on hematopoietic stem cell numbers and B cell differentiation. In the same vein, Horowitz and Lorenzo discuss the striking skeletal phenotypes of mice deficient in EBF-1 and Pax5, transcription factors with key roles in B cell differentiation. Teitelbaum and colleagues suggest new ways to think about the long-standing puzzle of glucocorticoid effects on bone and bone cells.

Taken together, the papers that make up this volume reflect much more than the inauguration of a convenient name for the field that lies at the intersection of skeletal biology and immunology. Since 1997, osteoimmunology has accumulated important insights, generated valuable tools, and attracted accomplished investigators from both traditional communities. To be sure, much remains unresolved and perhaps even unsuspected. In many instances, it remains unclear whether the skeletal and immune systems actually interact or are simply similar, deploying many of the same regulatory pathways, albeit to different ends. The question of the conditions under which lymphocytes can directly influence bone remodeling, already on the table in 1997, remains a topic of vigorous discussion. The immune system presents a very different face to the skeleton during infection or chronic inflammation than in a healthy individual. But at the very least, there is growing recognition that it can be unwise to design experiments or form conclusions in one traditional discipline without a thoughtful glance at the principles and lessons of the other. It may be that in future most bone biologists and most immunologists will be, to a greater or lesser degree, osteoimmunologists.

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CONTENTS

CHAPTER 1
SCHNURRI-3: A KEY REGULATOR OF POSTNATAL SKELETAL REMODELING

Dallas C. Jones[1,2], Marc N. Wein[1,2], and Laurie H. Glimcher[1]

1. ABSTRACT

Schnurri-3, a large zinc finger protein distantly related to Drosophila *Shn*, is a potent and essential regulator of adult bone formation. Mice lacking Shn3 display an osteosclerotic phenotype with profoundly increased bone mass due to augmented osteoblast activity. Shn3 controls protein levels of Runx2, the principal regulator of osteoblast differentiation, by promoting its degradation. In osteoblasts, Shn3 functions as a component of a trimeric complex between Runx2 and the E3 ubiquitin ligase WWP1. This complex inhibits Runx2 function and expression of genes involved in extracellular matrix mineralization due to the ability of WWP1 to promote Runx2 polyubiquitination and proteasome-dependent degradation. Our study reveals an essential role for Shn3 as a regulator of postnatal bone mass. Compounds designed to block Shn3/WWP1 function may be possible therapeutic agents for the treatment of osteoporosis.

2. INTRODUCTION

Bone is a dynamic tissue whose matrix components are continuously being turned over to preserve the structural integrity of the skeleton. Bone remodeling is a cyclical process where adult bone mass is determined by the rates of bone formation by osteoblasts and bone resorption by osteoclasts (Karsenty and Wagner 2002). Functional dysregulation of these cells affects bone mass and contributes to the pathogenesis of common skeletal disorders, like osteoporosis and Paget's disease. Rare single gene disorders resulting in elevated bone mass due to osteoclast defects are collectively termed osteopetrosis. Rarer still are single gene disorders, collectively termed osteosclerosis, in which elevated bone mass is due to intrinsically-elevated osteoblast activity (Whyte 2003).

Regulation of osteoblast differentiation and function involves a complex network of developmental cues and environmental factors. The transcription factor Runx2 interacts with a

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number of nuclear transcription factors, coactivators, and adaptor proteins in order to interpret these extracellular signals to appropriately regulate osteoblast differentiation (Stein, Lian, van Wijnen, et al. 2004). Runx2 is an essential component of skeletogenesis as mutations in Runx2 cause the human autosomal dominant disease cleidocranial dysplasia (Lee, Thirunavukkarasu, Zhou, et al. 1997; Mundlos, Otto, Mundlos, et al. 1997; Otto, Thornell, Crompton, et al. 1997). Runx2−/− mice exhibit a complete lack of both intramembranous and endochondral ossification, which results in an unmineralized skeleton (Komori, Yagi, Nomura, et al. 1997; Otto, Thornell, Crompton, et al. 1997).

In contrast to the significant progress in understanding the molecular mechanisms responsible for osteoblast differentiation during embryonic development, only a small number of genes are known to regulate postnatal osteoblast function. Runx2, in addition to its central role in osteoblast differentiation, also regulates osteoblast activity in adult mice (Ducy, Starbuck, Priemel, et al. 1999) in part through its induction of transcription factor ATF4, that regulates collagen biosynthesis in mature osteoblasts (Yang, Matsuda, Bialek, et al. 2004).

Schnurri-3 (Shn3) is one of three mammalian homologues of Drosophila *Shn*, a protein that acts during embryogenesis as an essential nuclear cofactor for Decapentaplegic (Dpp) signaling, the Drosophila homologue of BMP/TGF-ß (Affolter, Marty, Vigano, et al. 2001). While Shn3, a ZAS family protein, was originally identified as a DNA binding protein of the heptameric recombination signal sequence required for VDJ recombination of immunoglobulin genes (Wu, Mak, Dear, et al. 1993), we have described that it also functions as an adaptor protein in the immune system. Shn3 interacts with TRAF2 to inhibit NF-κB and JNK-mediated responses, including apoptosis and TNFα gene expression (Oukka, Kim, Lugo, et al. 2002). In T lymphocytes, Shn3 regulates IL-2 gene expression through its ability to partner with c-Jun and potentiate AP-1 transcriptional activity (Oukka, Wein, and Glimcher 2004). Although Shn3 has been demonstrated to regulate the activities of these important transcription factors *in vitro*, the *in vivo* role(s) of Shn3 remain largely unknown.

Here, we report on the essential role for Shn3 in regulating postnatal bone formation *in vivo* (Jones, Wein, Oukka, et al. 2006). Homozygous Shn3 mutant (Shn3−/−) mice exhibit a pronounced high bone mass phenotype, due to augmented osteoblast synthetic activity and bone formation. We present evidence that Shn3 functions in a multimeric complex comprised of Shn3, Runx2, and the Nedd4 family E3 ubiquitin ligase WWP1 in osteoblasts. This complex inhibits Runx2 function due to the ability of WWP1 to promote Runx2 polyubiquitination and proteasome-dependent degradation. Shn3 is an integral and required component of this complex, since its absence in osteoblasts results in elevated levels of Runx2 protein, enhanced Runx2 transcriptional activity, elevated transcription of Runx2 target genes, and profoundly increased rates of bone formation *in vivo*.

3. INCREASED BONE MASS IN Shn3−/− MICE

Digital radiographic analysis revealed increased radiopacity of the long bones of mature, 8 week old Shn3−/− mice (Figure 1A). Further analysis of skeletal architecture by μ-QCT revealed a dramatic increase in trabeculation within the long bones and vertebrae (Figure 1B-1C). Quantitative analysis revealed increases in both trabecular number and trabecular thickness in Shn3−/− femurs and vertebrae (Figure 1D-1E) resulting in a three-fold increase in trabecular bone volume (BV/TV) over WT (Figure 1F). The cortical thickness of Shn3−/− femurs and vertebrae was also slightly increased (Figure 1G). Additionally, the volumetric bone mineral density (vBMD) of Shn3−/− mice was >200% that of WT (Figure 1H).

To determine whether the elevated bone mass present in Shn3−/− mice was a result of abnormal prenatal bone development or dysfunction in postnatal skeletal bone formation, we analyzed

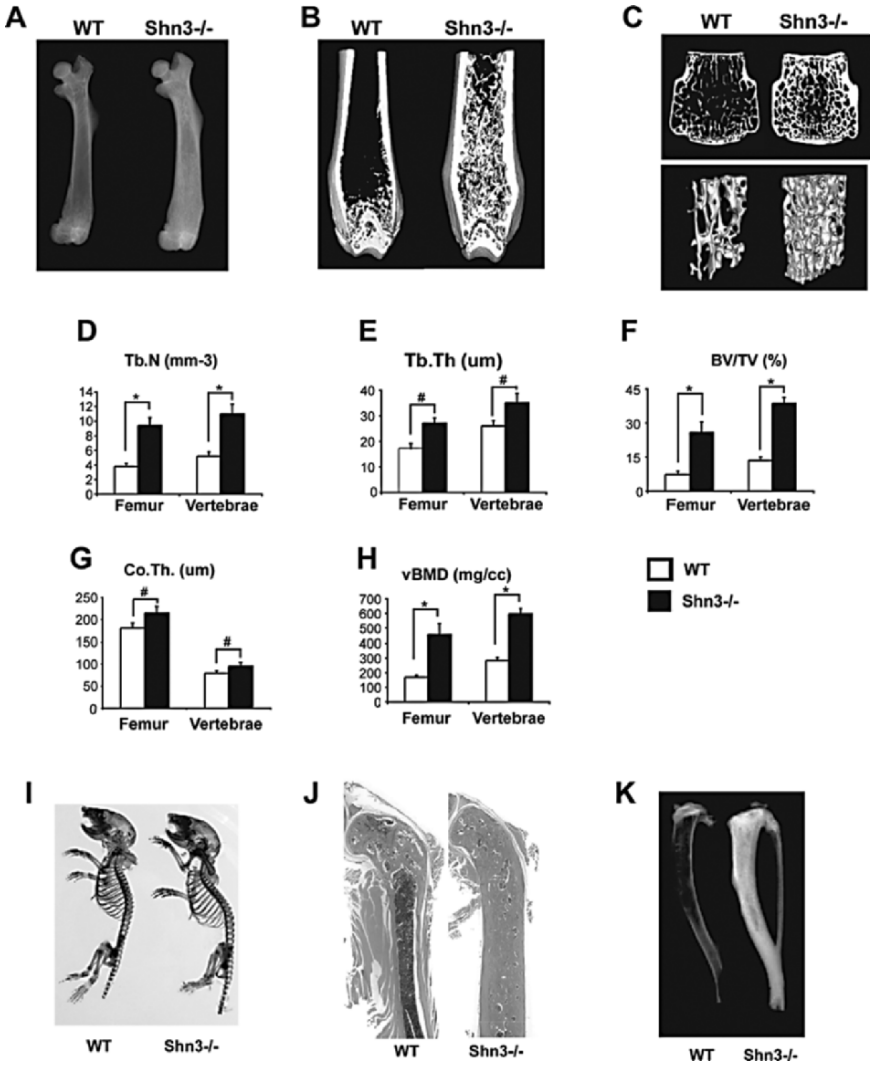

Figure 1. Shn3−/− mice exhibit increased bone mass. (A) Digital radiography of femurs isolated from 8-week-old WT and Shn3−/− mice. (B) Three-dimensional μ-QCT image of distal femurs isolated from Shn3−/− and WT control mice. (C) Two-dimensional (upper panel) and three-dimensional (lower panel) μ-QCT images of fourth lumbar vertebrae from WT and Shn3−/− mice. (D-H) Analysis of μ-QCT femur images from WT mice (open bars) and Shn3−/− mice (filled bars) for trabecular number (Tb.N.), trabecular thickness (Tb.Th.), bone volume per tissue volume (BV/TV), cortical thickness (Co.Th.) and volumetric bone mineral density (vBMD). (*$p<0.01$, $p<0.05$). (I) Alizarin red and alcian blue staining of Shn3−/− and WT mouse skeletons at P4. (J) Femurs from seven-month old Shn3−/− and WT mice were sectioned and stained with H&E. (K) Digital radiography of tibias isolated from 18-month-old WT and Shn3−/− mice. (Figures reprinted with permission from the Journal Science and the AAAS)

bone growth and development in newborn WT and Shn3−/− mice. Whole skeletal preparations from P4 WT and Shn3−/− mice were stained with alizarin red/alcian blue to analyze mineralized bone and non-mineralized cartilage formation, respectively. Figure 1I reveals that skeletal morphogenesis occurs normally in Shn3−/− mice analyzed at P4, with no premature cartilage mineralization detected

in those areas of the skeleton undergoing endochondral ossification. However, analysis at later time points revealed changes in the skeletal architecture of Shn3−/− mice between ages 1 and 2 weeks (Data not shown). The high bone mass phenotype we observed became noticeably more pronounced by seven months of age and was maintained in Shn3−/− mice as old as 18 months (Figure 1J-K).

4. Shn3 IS NOT REQUIRED FOR OSTEOCLAST DIFFERENTIATION OR FUNCTION

To investigate *Shn3*'s role in skeletal remodeling, we examined its expression in bone. Shn3 mRNA was detected in whole bone, osteoclasts, and, to a greater extent, in osteoblasts (Figure 2A). This nonrestrictive pattern suggested that the increased bone mass phenotype could result from alterations in osteoblast and/or osteoclast function. To detect possible *Shn3* function in osteoclasts, *in vitro* osteoclast differentiation assays were performed

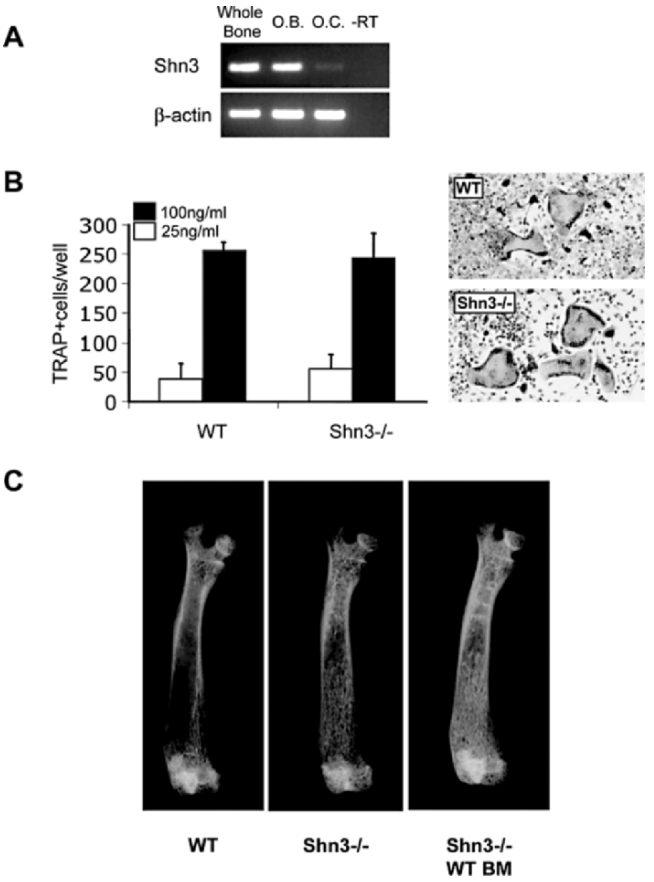

Figure 2. Shn3 expression is dispensable for osteoclast development and function. (A) Analysis of Shn3 mRNA expression in whole bone, osteoblasts (O.B.), osteoclasts (O.C.), and no reverse transcriptase control (-RT) by RT-PCR. (B) *In vitro* differentiation of osteoclast precursors from BM of WT and Shn3−/− mice in the presence of 50ng/ml M-CSF and 25ng/ml (open bars) or 100ng/ml (filled bars) RANKL. Cultures were then assayed for the presence of TRAP+ mature osteoclasts. (C) Radiographs of femurs from control WT mice, control Shn3−/− mice, and Shn3−/− mice four weeks after WT BM transfer. (Figures reprinted with permission from the Journal Science and the AAAS)

by culturing BM in the presence of M-CSF and RANKL to generate TRAP+ osteoclasts. Shn3−/− BM (Figure 2B) cultures generated similar numbers of multi-nucleated TRAP+ cells as compared to WT. Similar numbers of osteoclasts were also observed when WT and Shn3−/− splenocytes were cultured under conditions that promote osteoclastogenesis (data not shown). Thus *Shn3* expression is dispensable for osteoclast differentiation from precursor cells.

Skeletal abnormalities that result from osteoclast-intrinsic defects can be rescued following transfer of WT BM into irradiated hosts since WT donor osteoclasts, derived from hematopoietic progenitors, repopulate the host bone microenvironment and promote bone resorption (Li, Sarosi, Yan, et al. 2000). We transferred WT BM cells into lethally irradiated 4-week-old Shn3−/− mice and analyzed femurs by radiography after four weeks. Transfer of WT bone marrow failed to reduce the amount of trabeculation present in the femurs of recipient Shn3−/− mice (Figure 2C). These results further indicate that the increased calcified bone mass present in the Shn3−/− mice is not the result of deficiencies in the osteoclast lineage, but rather may result from increased osteoblast function and dysregulated bone formation.

5. ALTERED ACTIVITY OF Shn3−/− OSTEOBLASTS

To determine if the increased bone mass resulted from alterations in synthesis of bone matrix, we analyzed a number of histomorphometric parameters in 8-week old Shn3−/− and WT mice. Quantitative analysis revealed the bone formation rate (BFR) in Shn3−/− mice to be five-fold the rate observed in WT animals (Figure 3A). Additional histomorphometric analysis showed the Shn3−/− mice to have both an increased mineral apposition rate (MAR)

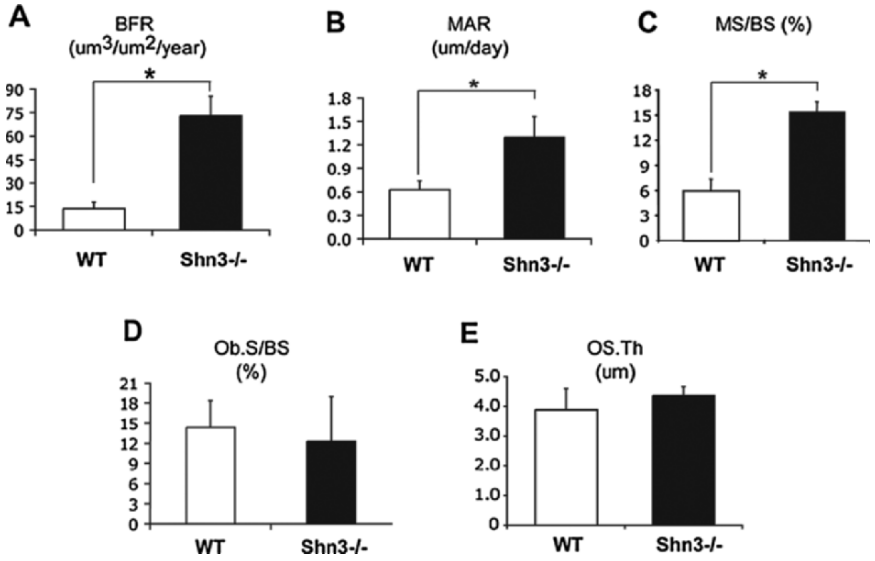

Figure 3. Increased Bone Formation Rate in Shn3−/− mice. (A-C) Bone formation rate (BFR) is significantly increased in Shn3−/− mice (filled bars) when compared to control WT mice (open bars). BFR is calculated by multiplying the mineral apposition rate (MAR), which is a reflection of the bone formation capabilities of osteoblasts, by the area of mineralized surface per bone surface (MS/BS) (*$p<0.02$). (D) The total osteoblast surface, a reliable indicator of osteoblast population, is similar between Shn3−/− and WT mice. (E) The osteoid thickness (OS.Th) in Shn3−/− mice is equivalent to the OS.Th observed in WT mice. (Figures reprinted with permission from the Journal Science and the AAAS)

and mineralizing surface (MS/BS) (Figure 3B-3C). The osteoblast surface (Ob.S/BS) in Shn3−/− mice (Figure 3F) and the thickness of the osteoid layer was comparable between WT and Shn3−/− mice (Figure 3G).

To verify that the increased bone mass observed in Shn3−/− mice arises from dysregulated osteoblast activity, we conducted a series of *in vitro* experiments on calvarial and bone marrow stromal derived osteoblasts from Shn3−/− and WT mice. Similar numbers of alkaline phosphatase positive cells were obtained from both Shn3−/− and WT osteoblast cultures (Figure 4A). However, Shn3−/− cultures had an increased number of mineralized nodules and these mineralized nodules were generally larger than those formed in the WT cultures (Figure 4B).

We next questioned if the increase in mineralized nodule formation by Shn3−/− osteoblasts resulted from alterations in the expression of genes involved in osteogenesis. Quantitative

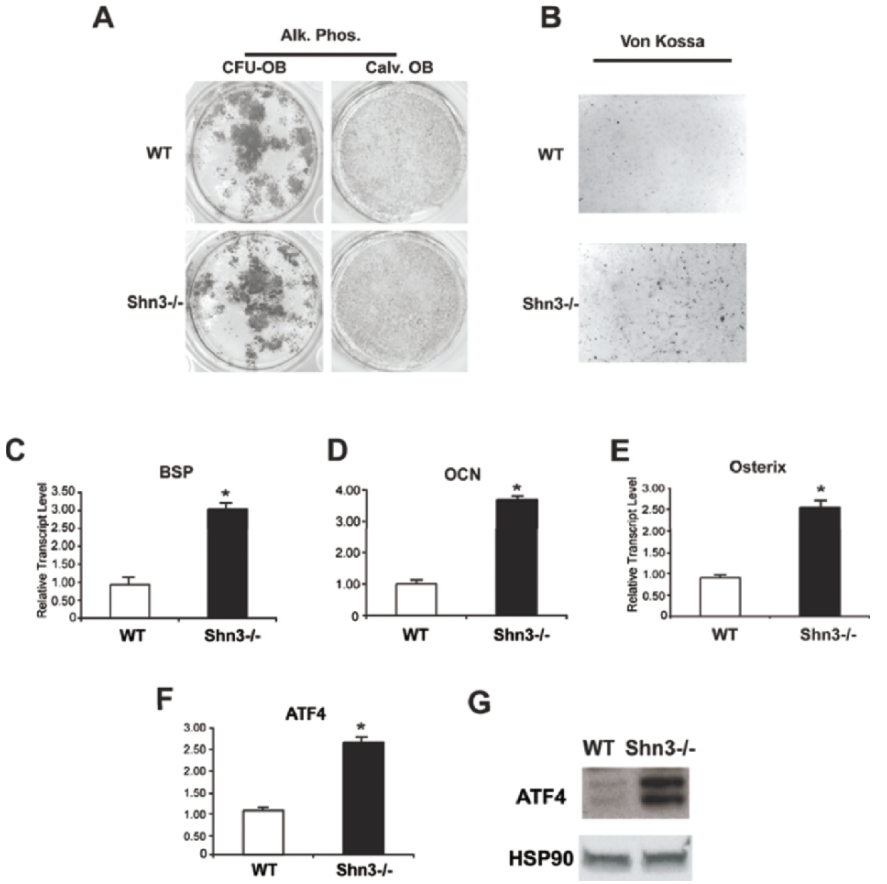

Figure 4. Analysis of Shn3−/− and WT osteoblast activity *in vitro*. (A) Similar number of alkaline phosphatase positive cells was observed in bone marrow stromal CFU-OB and calvarial osteoblast cultures isolated from Shn3−/− and WT mice. (B) Von Kossa staining shows mineralized nodule formation in calvarial osteoblast cultures derived from WT and Shn3−/− mice on day 21 of culture (C-E) Expression of bone sialoprotein (BSP), osteocalcin (OCN), and Osterix were analyzed in WT osteoblasts (open bars) and Shn3−/− osteoblasts (filled bars) by Q-PCR. The ordinate axis indicates the copy of mRNA per copy of β-actin mRNA. (F-G) WT and Shn3−/− osteoblasts were analyzed for the expression of ATF4 mRNA and protein by Q-PCR and Western blot, respectively. (Figures reprinted with permission from the Journal Science and the AAAS)

real-time PCR (Q-PCR) revealed Shn3−/− osteoblasts to express increased levels of BSP and OCN mRNA (Figure 4C-D). Shn3−/− osteoblasts also showed elevated levels of both Osterix and ATF4 mRNA and ATF4 protein, key regulators of osteoblast biology (Nakashima, Zhou, Kunkel, et al. 2002; Yang, Matsuda, Bialek, et al. 2004) (Figure 4E-G). Shn3 thus regulates the expression of a number of genes important in bone formation and mineralization.

6. Shn3 INTERACTS WITH Runx2 AND REGULATES ITS PROTEIN STABILITY

Since the osteoblast-specific genes overexpressed in Shn3−/− osteoblasts are all direct targets of Runx2 (Stein, Lian, van Wijnen, et al. 2004; Yang, Matsuda, Bialek, et al. 2004), we asked if Shn3 might exert its inhibitory influence on osteoblast activity *via* an effect on Runx2 itself. Interestingly, Shn3−/− osteoblasts contained elevated levels of Runx2 protein and Runx2 DNA binding even though levels of Runx2 mRNA were comparable between Shn3−/− and WT osteoblasts (Figure 5A-5C). This raised the possibility that Shn3 may regulate Runx2 protein stability. When overexpressed in 293T cells, Shn3 led to a dose-dependent decrease in steady-state Runx2 levels (Figure 5D). Furthermore, overexpression of Shn3 led to accelerated degradation kinetics of overexpressed Runx2, as judged by pulse-chase experiments (Figure 5E-F).

We reasoned that Shn3 may regulate Runx2 protein stability by physical interaction. Indeed, Runx2 specifically co-immunoprecipitated Shn3 in cotransfection studies, and this interaction was mediated via the Runt (DNA binding) domain of Runx2 (Figure 5G). We also detected an interaction between endogenous Runx2 and Shn3 in differentiated MC3T3-E1 osteoblastic cells (Zamurovic, Cappellen, Rohner, et al. 2004) (Figure 5H).

We next examined the consequences of the Runx2/Shn3 interaction with respect to Runx2 function. While Runx2 potently activated transcription from a multimerized OSE2-luciferase reporter construct, co-expression of Shn3 inhibited Runx2 activity in a dose-dependent manner (Figure 5I). Shn3 by itself had no effect on OSE2-luc activity (Figure 5I) nor did it bind to the OSE2 element (not shown). In addition to decreasing steady-state protein levels of Runx2, Shn3 inhibited binding of Runx2 to the canonical OSE2 Runx2 binding site (Figure 5J). From these studies, we conclude that Shn3 physically associates with Runx2, and this association leads to decreased Runx2 protein stability, as well as decreased Runx2 DNA binding and transactivation function.

7. Shn3 AND THE E3 LIGASE WWP1 PROMOTE THE UBIQUITINATION OF Runx2

Since Shn3 associates with and promotes the degradation of Runx2, we next asked whether Shn3 could promote the ubiquitination of Runx2. In overexpression studies, Shn3 promoted Runx2 ubiquitination (Figure 6A). Furthermore, when Shn3/Runx2 complexes were immunopurified from 293T cells and used in *in vitro* ubiquitination assays, specific ubiquitin ligase activity was detected (Figure 6B).

Shn3 promoted the ubiquitination of Runx2; however, Shn3 itself contains no canonical E3 ubiquitin ligase domains (RING, HECT, or U box, see (Patterson 2002; Pickart 2004) for review). Additionally, various recombinant protein fragments of Shn3 possessed no detectable *in vitro* E3 ubiquitin ligase activity (data not shown). These observations led us to hypothesize that Shn3 may associate with a known E3 ubiquitin ligase to promote Runx2 ubiquitination. It has

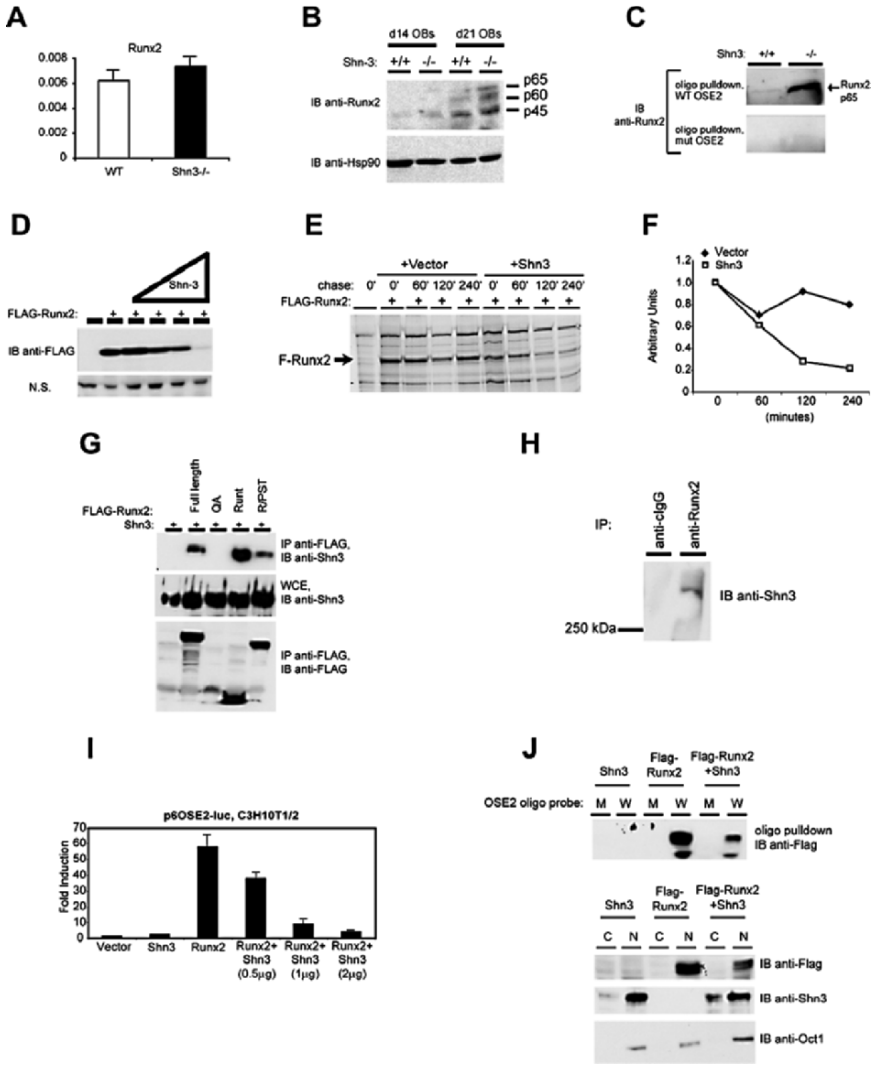

Figure 5. Stability and Activity of Runx2 is Inhibited through an Association with Shn3. (A) Runx2 mRNA levels are comparable in Shn3−/− and WT osteoblasts as determined by Q-PCR. (B) Western blot analysis of whole cell extracts generated from Shn3−/− and WT osteoblasts for levels of Runx2 protein present in osteoblasts at both day 14 and day 21 of culture. (C) Runx2 DNA binding was determined from nuclear extracts generated from WT or Shn3−/− osteoblats. Runx2 binding to either wild type (upper panel) or mutant (lower panel) OSE2 probe was detected by Western blotting following oligo pulldown experiments. (D) Runx2 protein levels were analyzed by Western blot in 293T cells following transfection with pFLAG-Runx2 and increasing levels of pEF-Shn3. Immunoblot was probed with anti-FLAG antibody to detect levels of FLAG-Runx2 protein. (N.S.-Non-specific loading control) (E-F) Degradation kinetics of Runx2 was analyzed by pulse-chase experiments in 293T cells transfected with pFLAG-Runx2 and control pEF-vector or pEF-Shn3. Panel E shows densitomery analysis of those bands corresponding to Runx2 protein. (G) Co-immunoprecipitation experiments were conducted in 293T cells with FLAG-epitope-tagged Runx2 deletion mutants. Full length (amino acids 1-521) contains QA, Runt and PST domains. QA mutant (amino acids 48-89) contains QA domain but lacks both Runt and PST domains. Runt mutant (amino acids 102-229) contains Runt domain only. Runt/PST mutant (amino acids 102-521) contains Runt and PST domain but lacks QA domain. Shn3 interaction with these mutants was determined by Western blot analysis with anti-Shn3 antibody following immunprecipitation with anti-FLAG antibody.

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previously been demonstrated that Runx2 could be ubiquitinated by over-expressed Smurf1, a member of the HECT domain-containing Nedd4 family of E3 ligases (Ingham, Gish, and Pawson 2004). However, Runx2 protein levels are normal in Smurf1-deficient mice, suggesting another Nedd4 family E3 ligase may regulate Runx2 ubiquitination *in vivo* (Yamashita, Ying, Zhang, et al. 2005).

We could readily co-immunoprecipitate Shn3 with WWP1 but we were unable to detect a physical interaction between Shn3 and Smurf1 or Smurf2 (Figure 6C, left). Additionally, endogenous WWP1 immunoprecipitates from differentiated MC3T3-E1 cells reproducibly contained 95 kDa Shn3 immunoreactive species (Figure 6C, right).

Since Shn3 and Runx2 physically interact (Figure 5G-5H), and Shn3 and WWP1 interact (Figure 6C), we next asked whether Runx2 and WWP1 associate. When overexpressed in 293T cells, we detected an interaction between Runx2 and WWP1. Moreover, this interaction was enhanced by coexpression of Shn3 (Figure 6D-left). Furthermore, deletion mapping studies defined the Runt domain of Runx2 as the minimal domain required for interaction with WWP1 (Figure 6D-right). An interaction between endogenous WWP1 and Runx2 p65 isoform was also detected in ROS 17/2.8 cells (Figure 6E). WWP1 overexpression led to dose-dependent reductions in steady-state levels of Runx2 protein, which was reversed by brief treatment with the proteasome inhibitor MG132 (Figure 6F). Finally, WWP1 promoted low levels of Runx2 ubiquitination when overexpressed in 293T cells. However, when WWP1 was coexpressed with Shn3, the two synergistically acted to promote Runx2 ubiquitination (Figure 6G).

8. WWP1 IS A POTENT INHIBITOR OF OSTEOBLAST FUNCTION

To further investigate the role of WWP1 in osteoblasts, we utilized lentiviruses (LV) harboring RNAi constructs designed to reduce levels of endogenous WWP1. We transduced MC3T3-E1 cells with lentiviruses expressing RNAi against WWP1 (WWP1-i) or control RNAi against Itch (Itch-i), and GFP (GFP-i). RNAi-mediated knockdown of WWP1, but not the related E3 ligase Itch, led to dramatic upregulation of osteoblast markers ALP, BSP, OCN, and ATF4 at the mRNA level in MC3T3-E1 cells (Figure 7A). Additionally, levels of Runx2 mRNA were similar between GFPi and WWP1i-expressing cells but Runx2 protein levels were increased in WWP1i cultures (Figure 7B).

Finally, primary calvarial osteoblasts transduced with RNAi-expressing lentiviruses against either WWP1 (results for two independent constructs are shown) or against GFP during the course of *in vitro* differentiation were analyzed for the expression of genes involved in osteogenesis as well as matrix mineralization. Much like Shn3-deficient osteoblasts (Figure 4), WWP1 knockdown osteoblasts showed dramatic upregulation of Runx2 targets BSP, OCN, and ATF4 at the mRNA level (Figure 7C). Also, WWP1 knockdown cultures showed elevated levels of Runx2 protein (Figure 7D) while Runx2 mRNA levels were comparable between WWP1

Figure 5. cont'd. (H) Co-immunoprecipitation experiments were conducted in BMP2-differentiated MC3T3-E1 cells. Runx2 protein was immunoprecipitated from cell lysates with anti-Runx2 antibody, followed by Western blot analysis with anti-Shn3 antibody to detect Shn3 interaction. (I) C3H10T1/2 cells were transfected with p6xOSE2-Luc reporter, a Runx2 expression plasmid and increasing amounts of the Shn3 expression plasmid. Results were normalized to the expression of the pRL-TK plasmid. (J) Runx2 DNA binding in the presence or absence of co-transfected Shn3 was determined from nuclear extracts of 293T cells transfected as indicated. Runx2 binding to either mutant (M) or wild type (W) OSE2 probe was detected as described in (C). Overall levels of Runx2 in cytosolic or nuclear fractions are shown below. (Figures reprinted with permission from the Journal Science and the AAAS)

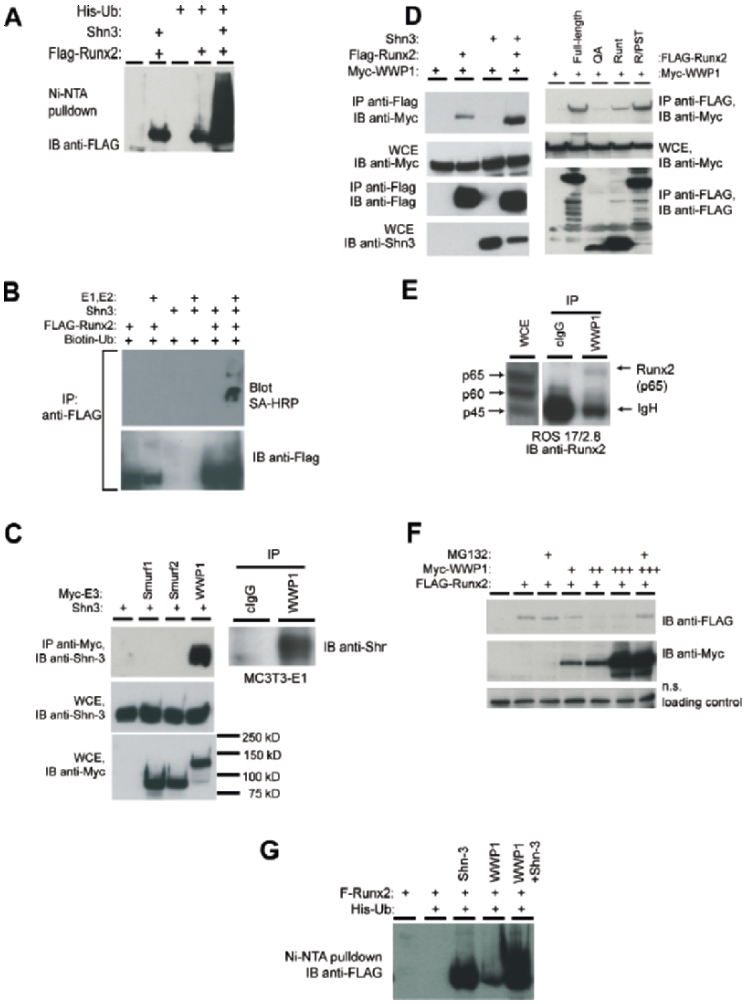

Figure 6. Shn3 Interacts with WWP1 to Enhance the Ubiquitination of Runx2. (A) Analysis of Runx2-ubiquitination was performed in 293T cells transfected with combinations of Shn3, FLAG-epitope-tagged Runx2, and His-tagged ubiquitin. Ubiquitinated FLAG-Runx2 proteins were detected in cells by precipitating ubiquitinated proteins from denatured lysates with Ni-NTA-agarose, followed by Western blot analysis with anti-FLAG antibody to detect ubiquitinated Runx2 protein. (B) *In vitro* ubiquitination assays were performed on lysates from 293T cells that were transfected with FLAG-Runx2 and/or Shn3. FLAG-epitope-tagged proteins immunoprecipitated from transfected cell lysates were incubated in vitro with ubiquitin, biotinylated-ubiquitin, and recombinant E1 and E2. Reactions were then subjected to Western blot analysis with streptavidin-HRP to dectect the presence of biotin-ubiquitinated proteins. (C) **Left**, Analysis of association between Shn3 and three members of HECT domain-containing E3 ligases was conducted in 293T cells transfected with pEF-Shn3 and Myc-Smurf1, Myc-Smurf2, or Myc-WWP1. Myc-epitope tagged proteins were immunoprecipitated from cell lysates with anti-myc antibody, followed by Western blot analysis with anti-Shn3 antibody to detect Shn3 interaction. **Right**, MC3T3-E1 cells were differentiated *in vitro*, endogenous WWP1 was immunoprecipitated, followed by SDS-PAGE and anti-Shn3 immunoblotting. (D) **Left**, Analysis of association between WWP1 and Runx2. 293T cells were transfected as indicated, followed by anti-FLAG (Runx2) immunoprecipitation and anti-Myc (WWP1) immunoblotting. **Right**, 293T cells were transfected with Myc-WWP1 and various deletion mutants of Runx2 as described in Figure 5G. Runx2 was immunoprecipitated with anti-FLAG followed by anti-Myc (WWP1) immunoblotting. (E) ROS 17/2.8 cell lysates were immunoprecipitated with anti-WWP1 antibodies, and endogenous Runx2 proteins in

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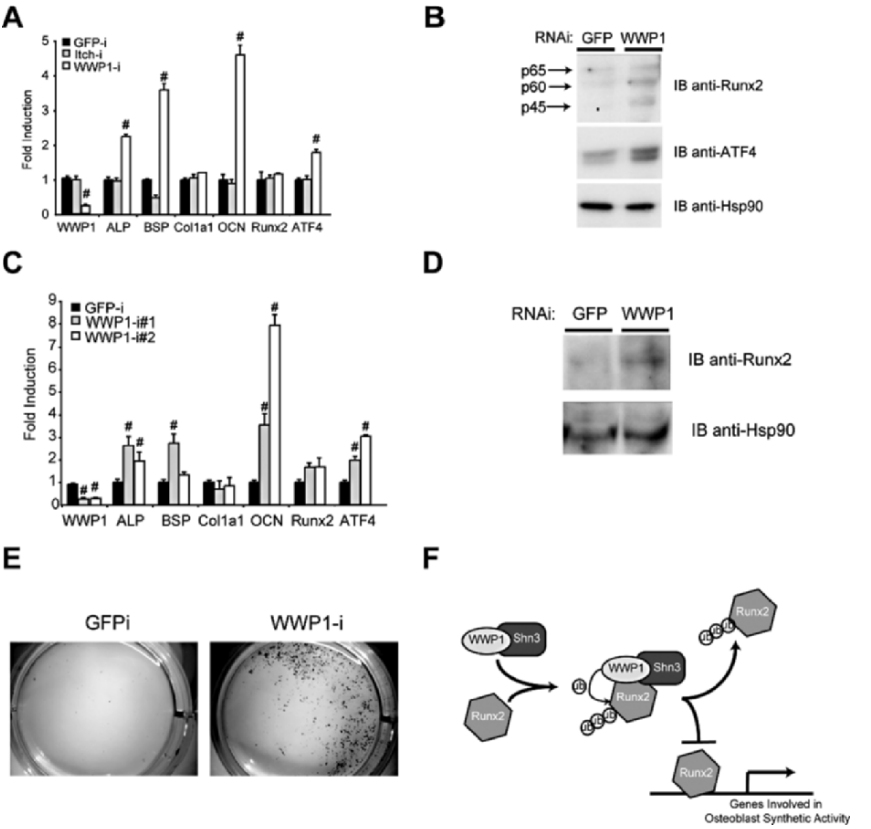

Figure 7. WWP1 Inhibits Runx2-driven Transcription in Osteoblasts. (A) RNAi-expressing MC3T3-E1 cells were differentiated with BMP2/ascorbic acid/ß-glycero-phosphate and expression of the indicated genes was analyzed by Q-PCR. Values are shown relative to GFPi-expressing cells (#$p<0.05$). (B) Lysates from MC3T3-E1 cells in part were analyzed by immunoblotting for Runx2, ATF4, and Hsp90 protein levels. (C) RNAi-expressing primary calvarial osteoblasts were analyzed for expression of the indicated genes by Q-PCR. Values are shown relative to GFPi-expressing cells (#$p<0.05$). (D) Lysates from cells in part (C) were analyzed by immunoblotting for Runx2 and Hsp90 protein levels. (E) Osteoblasts expressing RNAi against GFP or WWP1 were cultured for 14 days, followed by von Kossa staining to reveal the presence of mineralized matrix nodules. (F) Proposed model: formation of a complex between Shn3, WWP1, and Runx2 in mature osteoblasts leads to the ubiquitination and down-regulation of Runx2, a transcription factor key in the expression of genes involved in extracellular matrix mineralization. (Figures reprinted with permission from the Journal Science and the AAAS)

Figure 6. cont'd. immunoprecipiates were determined by immunoblotting. (F) Runx2 protein levels following co-transfection of increasing amounts of WWP1. Where indicated, 293T cells were pre-treated with MG132 (10 μM) for one hour prior to lysis. 40 μg total protein was resolved by SDS-PAGE followed by immunoblotting as indicated. (G) Analysis of Runx2-ubiquitination was performed in 293T cells transfected with FLAG-Runx2 and combinations of Myc-WWP1 and Shn3 as described in (A). (Figures reprinted with permission from the Journal Science and the AAAS)

RNAi cells and controls. Finally, WWP1 knockdown cultures showed increased numbers of mineralized matrix nodules (Figure 7E).

9. CONCLUSION

The present study establishes a role for Shn3 as a potent inhibitor of bone formation via an osteoblast-intrinsic mechanism. Taken together, these data suggest a model (Figure 7F) in which Shn3 regulates osteoblast function through an association with WWP1 to regulate the ubiquitination and hence degradation of Runx2. Our observation that Shn3 inhibits Runx2 function in osteoblasts to regulate bone formation *in vivo* suggests that compounds designed to block Shn3/WWP1 function may be possible therapeutic agents for the treatment of osteoporosis.

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CHAPTER 2
ECTODOMAIN SHEDDING OF RECEPTOR ACTIVATOR OF NF-κB LIGAND

Atsuhiko Hikita[1] and Sakae Tanaka[2]

1. ABSTRACT

Receptor activator of NF-κb ligand (RANKL), a key regulator of osteoclastogenesis, is proteolytically processed and converted to a soluble form. RANKL sheddase and the biologic and pathologic role of RANKL shedding have been undetermined, but the identity of sheddase and its effect on osteoclastogenesis are gradually clarified. The regulatory mechanism and its relevance to some pathologic conditions are to be elucidated.

2. INTRODUCTION

Ectodomain shedding is a tightly regulated process which releases the extracellular domain of membrane-bound proteins. It affects the biological and pathological activities of various proteins in diverse ways. For example, amyloid precursor protein is cleaved at different sites. When it is processed by α-secretases, such as ADAM9, ADAM10 and ADAM17, a neurotrophic protein APPsα is produced (Koike, Tomioka, Sorimachi, et al. 1999; Lammich, Kojro, Postina, et al. 1999; Buxbaum, Liu, Luo, et al. 1998). In contrast, its cleavage by β-secretase, BACE, followed by an intramembrane proteolysis by γ-secretase, produces APPsβ, which is a primary constituent of the senile plaques in brains from Alzheimer's patients (Vassar, Bennett, Babu-Khan, et al. 1999; Li, Lai, Xu, et al. 2000; Selkoe 1991).

Receptor activator of NF-κB ligand (RANKL) is a type II transmembrane protein of tumor necrosis factor (TNF) ligand family (Figure 1). RANKL is expressed on the plasma membrane of primary osteoblasts, bone stromal cells and T lymphocytes (Anderson, Maraskovsky, Billingsley, et al. 1997; Wong, Rho, Arron, et al. 1997; Yasuda, Shima, Nakagawa, et al. 1998; Lacey, Timms, Tan, et al. 1998). Critical roles of RANKL in osteoclast

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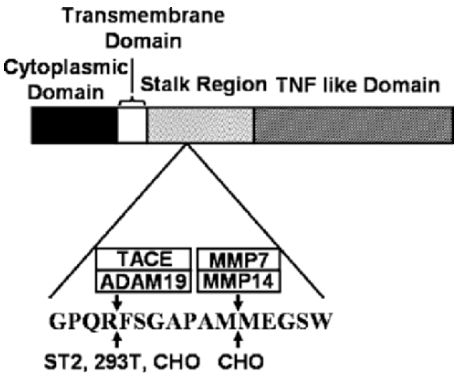

Figure 1. A schematic representation of RANKL. RANKL is consisted of cytoplasmic domain, transmembrane domain, stalk region and TNF like domain. RANKL is proteolytically processed at stalk region. The reported cleavage sites are indicated by arrows. ST2, 293T and CHO means the cleavage sites by endogenous proteinases in these cells.

differentiation, activation and survival is clarified by several lines of mice which lack RANKL or its receptor RANK (Kong, Yoshida, Sarosi, et al. 1999; Dougall, Glaccum, Charrier, et al. 1999). Like other members of TNF ligand family such as TNF-α and Fas ligand, RANKL is also cleaved and released into the local environment as well as the general circulation (Lum, Wong, Josien, et al. 1999; Nakashima, Kobayashi, Yamasaki, et al. 2000).

3. CANDIDATES OF RANKL SHEDDASE

Several proteinases have been considered as RANKL sheddases. Studies using specific inhibitors have suggested that the RANKL sheddase may be a member of matrix metalloproteinases (MMPs) or a disintegrin and a metalloproteinases (ADAMs) (Lum, Wong, Josien, et al. 1999; Nakashima, Kobayashi, Yamasaki, et al. 2000). ADAM17, also called as TNF alpha converting enzyme (TACE), was the first candidate of RANKL sheddase because RANKL and TNF-α belong to the same superfamily (Lum, Wong, Josien, et al. 1999). TACE cleaved a RANKL peptide in vitro. But a subsequent report from the same group showed that TACE is unable to cleave the full-length RANKL in cell-based assay and RANKL shedding is similarly observed in TACE-deficient embryonic fibroblasts (Schlondorff, Lum, and Blobel 2001). In this report, MMP14, also called as membrane-type 1 matrix metalloproteinase (MT1-MMP), was shown to cleave RANKL, but its cleavage site was different from that previously reported in ST2 cells or 293T cells (Lum et al. 1999; Nakashima, Kobayashi, Yamasaki, et al. 2000). In 2003, RANKL shedding activity of ADAM19 was demonstrated, but RANKL shedding also occurred in ADAM19 deficient embryonic fibroblasts (Chesneau, Becherer, Zheng, et al. 2003). Recently, Lynch et al. reported that prostate cancer cells are able to process RANKL to a soluble form that promotes osteoclast activation, which is mediated by MMP7 (Lynch, Hikosaka, Acuff, et al. 2005). We established a screening system for RANKL sheddase (s) and found MMP14 as a positive clone from the cDNA library of a bone marrow stromal cell line, ST2. We also showed that MMP14 was the endogenous RANKL sheddase in human osteosarcoma cell line, SaOS2 by RNA interference (Hikita, Kadono, Chikuda, et al. 2005). Recently, we identified MMP14 as the endogenous RANKL sheddase in primary osteoblasts (Hikita, Yana, Wakeyama, et al. 2006).

4. BIOLOGIC AND PATHOLOGIC MEANINGS OF RANKL SHEDDING

The physiologic and pathologic influences of shedding of other two members of TNF family, TNF-α and Fas ligand, are quite different. Release of TNF-α from the plasma membrane via ectodomain shedding results in the systemic effect of the molecule (Kriegler, Perez, DeFay, et al. 1988; Black, Rauch, Kozlosky, et al. 1997). On the contrary, Fas ligand has much weaker activity in its soluble form (Schneider, Holler, Bodmer, et al. 1998). As for RANKL, the significance of its ectodomain shedding is still controversial (Figure 2). It has been reported that the concentration of soluble RANKL in the joint fluid or serum of rheumatoid arthritis (RA) patients is higher than that of osteoarthritis or healthy control although it may just reflect the higher expression level of membrane-bound RANKL in the patients (Kotake, Udagawa, Hakoda, et al. 2001; Ziolkowska, Kurowska, Radzikowska, et al. 2002). The promoted shedding of RANKL in RA patients may be due to the high level expression of MMP14 in the synovium compared to healthy control (Yamanaka, Makino, Takizawa, et al. 2000). Soluble RANKL produced in the patients may cause systemic bone loss by promoting osteoclast formation, but it is also possible that ectodomain shedding may work as a negative regulator of local bone resorption by reducing membrane-bound RANKL.

4.1. Downregulation of Local Osteoclastogenesis

Before the cloning of RANKL by several groups, it was speculated that some osteoclastogenic factor(s) are expressed in osteoblasts, and the necessity of direct contact of osteoblasts and osteoclast precuosor was suggested by the culture of these two cells with or without mem-

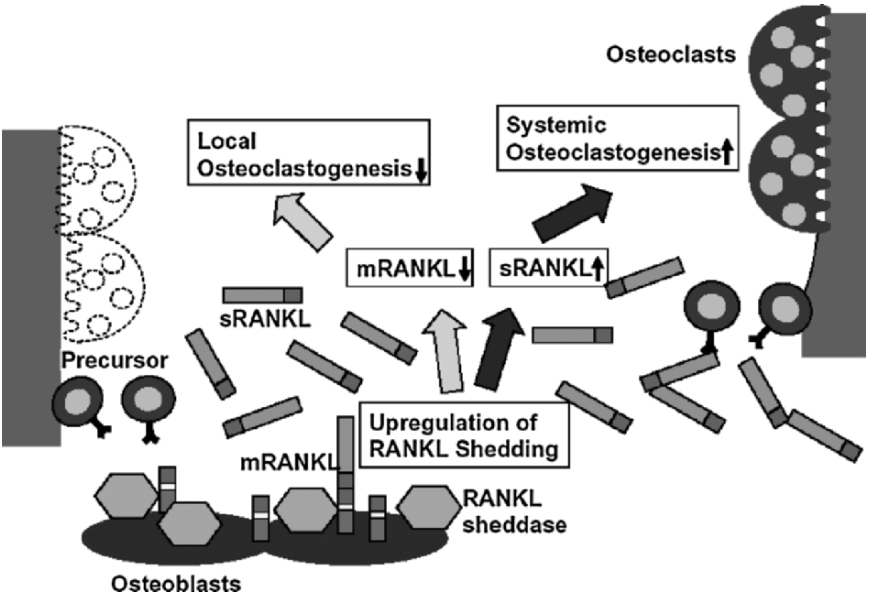

Figure 2. Schematic representation of the hypothesis of the effects of RANKL shedding on osteoclastogenesis. The left half of the figure represents the concept of downregulation of RANKL, and the right represents the remote effect of soluble RANKL. mRANKL, membrane-bound RANKL; sRANKL, soluble RANKL.

brane filter (Takahashi, Akatsu, Udagawa, et al. 1988). Nakashima et al. showed that membrane bound RANKL was more potent to induce osteoclastogenesis than soluble RANKL (Nakashima, Kobayashi, Yamasaki, et al. 2000). In the coculture of bone marrow cells and SaOS-4/3 which were derived from SaOS2, osteoclastogenesis occurred only in the area where SaOS-4/3 cells were spotted (Itoh, Udagawa, Matsuzaki, et al. 2000). Taking these reports into account, RANKL shedding would be a process that weakens the local osteoclastogenic activity of osteoblasts/ stromal cells. In the report of Holmbeck et al., MMP14-deficient mice showed osteopenia and increased osteoclastogenesis (Holmbeck, Bianco, Caterina, et al. 1999). We identified MMP14 as endogenous RANKL sheddase in an osteosarcoma cell line, SaOS2, then it seems to be plausible that the lack of MMP14 increases membrane-bound RANKL which has stronger activity than soluble one, and upregulates osteoclastogenesis in MMP14 deficient mice in vivo (Hikita, Kadono, Chikuda, et al. 2005). Recently, our group found that suppression of RANKL shedding in primary osteoblasts by siRNA construct for MMP14 increases membrane-bound RANKL and promotes osteoclastic activity of osteoblasts in coculture with osteoclast precursors (Hikita, Yana, Wakeyama, et al. 2006).

4.2. Remort Effect of Soluble RANKL on Osteoclastogenesis

Several reports suggest that the release of soluble RANKL has paracrine effects like that of TNF-α. Transgenic mice overexpressing soluble RANKL showed osteoporosis (Mizuno, Kanno, Hoshi, et al. 2002). Solubilization of RANKL was suggested to cause osteolysis in prostate cancer cells (Lynch, Hikosaka, Acuff, et al. 2005). However, it is uncertain whether the concentration of the soluble RANKL created by shedding is sufficient to induce osteoclastogenesis. Actually, recombinant soluble RANKL is often used for osteoclast formation in vitro, but the concentration required is more than 10 ng/ml (Yasuda et al. 1998). The concentration of RANKL in the mouse serum or culture medium of osteoblasts stimulated by 1α, $25(OH)_2D_3$, PGE_2 is usually no more than 1 ng/ml (Nakamura, Udagawa, Matsuura, et al. 2003). Still it is possible that soluble RANKL has osteoclastogenic effects in vivo in some pathological conditions, for example, in the local environments where RANKL is sufficiently concentrated or in the presence of TNF-α, which works synergistically with RANKL on osteoclastogenesis (Lam, Takeshita, Barker, et al. 2000).

4.3. Other Effects of RANKL Shedding

RANKL was identified as a factor which enhanced T-cell growth and dendritic cell function at first, and RANKL also has an effect on mammalian gland growth during pregnancy (Anderson, Maraskovsky, Billingsley, et al. 1997; Fata, Kong, Li, et al. 2000).

Recently, the involvement of RANKL in the metastasis of human epithelial cancer cells and melanoma cells that express the receptor RANK was proposed (Jones, Nakashima, Sanchez, et al. 2006). These tumor cells express RANK, and RANKL stimuli induce actin polymerization, and causes cell migration. Migration of mature osteoclasts was also shown in the same report, although the effect of RANKL on the tumor cells was independent of osteoclasts. Whether RANKL shedding causes chemotaxis of tumor cells and osteoclasts or reduces the entrapment of circulating tumor cells to bone milieu is to be determined.

5. REGULATION OF RANKL SHEDDING

There were few reports which focused on RANKL shedding, and the regulation of RANKL shedding has largely remained elusive. Several proinflammatory cytokines such as interleukin (IL) -1, a combination of IL-6 and its soluble receptor IL-6R, IL-11, IL-17 and TNF-α increased soluble RANKL in the culture media of mouse primary osteoblasts, but this may just reflect their stimulatory effects on the expression of membrane-bound RANKL (Nakashima, Kobayashi, Yamasaki, et al. 2000). Pervanadate (tyrosine phosphatase inhibitor), but not PMA (phorbol ester, protein kinase C activator), stimulated RANKL shedding in CHO cells (Schlondorff, Lum, and Blobel 2001). Using a RANKL shedding screening assay as described above, we identified alternatively spliced variant of Ca^{2+}-promoted Ras inactivator (ΔCAPRI) as an intracellular modulator of RANKL shedding. CAPRI was originally identified as a member of the RasGAP, negative regulators of Ras signaling pathways. ΔCAPRI has a deletion in the GAP domain of CAPRI, and positively regulates Ras signaling probably by working in a dominant negative fashion, and the activation of Ras signaling pathway promoted RANKL shedding in 293T cells (Hikita, Kadono, Chikuda, et al. 2005).

Regulation of MMP14, which is the most plausible endogenous RANKL sheddase, was shown in several reports. Progesterone or 17β-estradiol increased the expression of MMP14 in osteoblastic cell line, MG-63 (Luo and Liao 2001; Liao, Luo, Deng, et al. 2001). Parathyroid hormone not only increased the expression of RANKL, but also suppressed the expression of MMP14 in human osteoblasts or MG-63, which might result in an effective expression of membrane-bound RANKL (Guo, Xie, Zhou, et al. 2004; Luo, Liao, Su et al. 2004).

6. CONCLUSION

Endogenous RANKL sheddases(s) and the biologic roles of RANKL shedding are gradually elucidated. The activity of soluble RANKL in pathologic conditions such as inflammation or bone metastasis of tumors needs to be clarified. Regulatory mechanisms of RANKL shedding remains largely unknown, but their elucidation is quite important to clarify the control mechanism of the osteoclastogenesis in local environment or systemic bone milieu.

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CHAPTER 3
THE NEGATIVE ROLE OF IDS IN OSTEOCLASTOGENESIS

Junwon Lee[1], Seoung-Hoon Lee[2], Yongwon Choi[2], and Nacksung Kim[1]

1. INTRODUCTION

Osteoclasts play an important role in bone metabolism by resorbing the bone matrix. These cells originate from hematopoietic precursors and share a common progenitor with macrophages and dendritic cells (DCs). Two essential cytokines, macrophage colony-stimulating factor (M-CSF) and TRANCE (also called RANKL, OPGL, and ODF), enable osteoclast differentiation from their monocyte/macrophage lineage precursors (Suda, Takahashi, Udagawa, et al. 1999; Yasuda, Shima, Nakagawa et al. 1998; Lacey, Timms, Tan, et al. 1998).

TRANCE, a TNF family member, supports osteoclast differentiation, survival, and activation. Binding of TRANCE to its receptor, receptor activator of nuclear factor κB (RANK), activates multiple signaling pathways mediated by TNF receptor-associated factors (TRAFs), including NF-κB, c-Jun N-terminal kinase (JNK), p38 MAP kinase, extracellular signal-related kinase (ERK), and AKT (Lee and Kim 2003; Boyle, Simonet and Lacey 2003).

It has been shown that TRANCE induces activation and/or induction of transcription factors such as Mitf, PU.1, and NFATc1 (Boyle, Simonet and Lacey 2003; Teitelbaum 2000; Teitelbaum and Ross 2003). Mitf is known to be important for osteoclastogenesis *in vitro* and *in vivo* (Holtrop, Cox, Eilon, et al. 1981; Thesingh and Scherft 1985; Luchin, Purdom, Murphy, et al. 2000).TRANCE activates Mitf via the MKK6/p38 signaling cascade. Subsequently, activated Mitf induces the expression of target genes, including TRAP, cathepsin K, and OSCAR, which are important for osteoclast differentiation or function (Luchin, Purdom, Murphy, et al. 2000; Motyckova, Weilbaecher, Horstmann, et al. 2001; Mansky, Sankar, Han, et al. 2002; So, Rho, Jeong, et al. 2003; Kim, Takami, Rho, et al. 2002), by binding to the canonical E-box sequence in the promoter region of those genes.

The HLH family of transcriptional regulatory proteins have important roles in developmental processes including neurogenesis, myogenesis, and hematopoiesis (Massari and Murre 2000). Tissue-specific bHLH proteins form dimers with E proteins, ubiquitously expressed bHLH transcription

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factors, which bind to the E-box sequence CANNTG. HLH proteins can bind to bHLH transcription factors via their HLH domain; the resulting heterodimers are unable to bind to DNA because HLH proteins lack the necessary basic motif (Benezra, Davis, Lockshon, et al. 1990). Thus, HLH proteins act as dominant negative regulators of bHLH transcription factors. One of the HLH subfamilies is composed of Id genes 1 to 4. Id genes are thought to affect the balance between cell growth and differentiation in many cell types, including osteoblasts, keratinocytes, myoblasts, and mammary epithelial cells. Differentiation of various cell lineages is shown to be accompanied by the downregulation of Id expression, while overexpression of Id genes in cultures of mammalian cells is shown to inhibit their ability to differentiate under appropriate conditions (Lasorella, Uo and Iavarone 2001). Although Id genes regulate cell proliferation and differentiation in many cell types, the role of Id genes in osteoclast differentiation has not been determined.

2. DOWNREGULATION OF IDS BY TRANCE

TRANCE induces activation and induction of transcription factors including NF-κB, c-fos, Mitf, and NFATc1, and thereby supports osteoclast differentiation from precursors (Boyle, Simonet and Lacey 2003; Teitelbaum 2000; Teitelbaum and Ross 2003). To identify important genes for osteoclast differentiation, we performed PCR-select™ cDNA subtraction between osteoclasts and macrophages derived from common precursors (BMMs), and found that Id2 was downregulated in osteoclasts compared to macrophages. To confirm the differential expression of the Id2 gene in both cells, we examined the expression pattern of Id genes during TRANCE-mediated osteoclastogenesis. Among the four known members of the Id family, Id1, Id2, and Id3 are expressed in the RAW264.7 cell line, which is derived from the monocyte/macrophage lineage and is capable of differentiating into osteoclasts and resorbing bone. The expression of these genes was significantly reduced 1 h after TRANCE treatment and was maintained at that low level throughout the osteoclastogenic process, whereas the expression of TRAP, a marker for mature osteoclasts, increased as the cells differentiated (Figure 1). However, Id4 was not detected in any stage of osteoclastogenesis mediated by TRANCE (data not shown).

Mitf, a bHLH transcription factor, is known to regulate osteoclastogenesis mediated by TRANCE (Holtrop, Cox, Eilon, et al. 1981; Thesingh and Scherft 1985; Luchin, Purdom, Murphy, et al. 2000). It has been shown that TRANCE signaling in osteoclasts results in Mitf phosphorylation mediated by a MKK6/p38 signaling cascade and also induces expression of the Mitf gene (Mansky, Sankar, Han et al. 2002; So, Rho, Jeong, et al. 2003). HLH transcription factors such as Id genes form heterodimers with members of the bHLH family of transcription factors

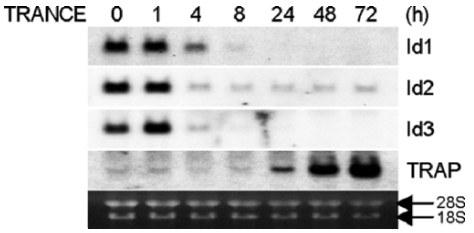

Figure 1. TRANCE downregulates the expression of Id genes during osteoclastogenesis. RAW264.7 cells were cultured for the indicated time in the presence of TRANCE. Total RNA was collected from each time point and analyzed by Northern blot using probes for Id1, Id2, Id3, and TRAP.

and regulate cell growth and differentiation (Norton 2000; Sikder, Devlin, Dunlap, et al. 2003). They act as dominant-negative inhibitors of their binding partners. Previously, we showed that TRANCE treatment increased Mitf expression in RAW264.7 cells and in BMMs (So, Rho, Jeong, et al. 2003). These results suggest that TRANCE signaling regulates the expression of bHLH and HLH transcription factors during osteoclast differentiation and that the balance of expression of these genes may be important for the process of osteoclastogenesis.

3. THE NEGATIVE ROLE OF IDS ON OSTEOCLASTOGENESIS

To investigate the role of Id genes in osteoclastogenesis mediated by TRANCE, we over expressed Id genes in BMMs using a retroviral vector. Transduced BMMs were cultured with M-CSF alone or M-CSF and TRANCE, and were stained for TRAP. Although a small number of TRAP(+) mononuclear cells were detected, overexpression of Id1, Id2, or Id3 gene largely inhibited the formation of TRAP(+) MNCs mediated by M-CSF and TRANCE, whereas control vector-infected BMMs could mature into osteoclasts (Figure 2A). We confirmed that overexpression of Id genes did not affect the proliferation or survival of BMMs by means of an MTT assay (data not shown). These data suggest that Ids play a negative role in TRANCE-mediated osteoclastogenesis.

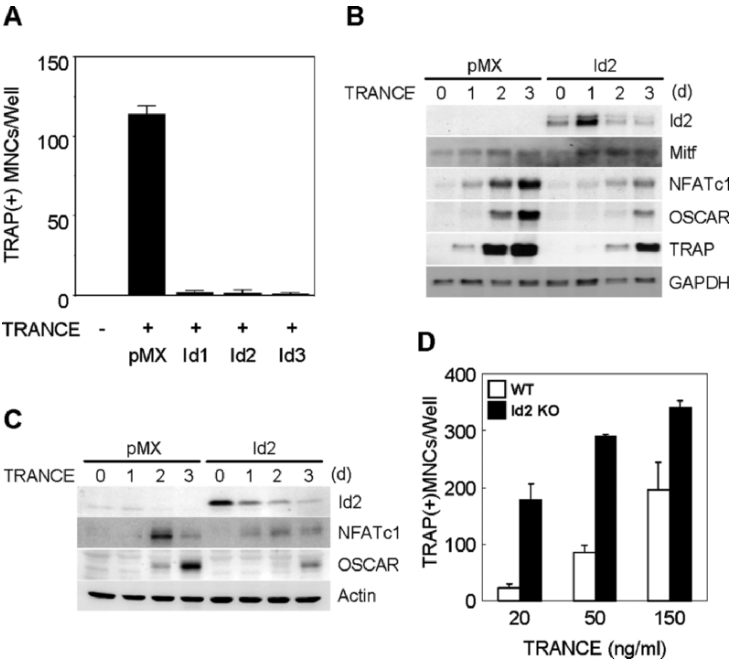

Figure 2. The negative role of Ids in TRANCE-mediated osteoclastogenesis. (A) BMMs were transduced with pMX-IRES-EGFP (control), Id1, Id2, or Id3 retrovirus and cultured for 3 days with M-CSF alone or M-CSF and TRANCE. Cultured cells were fixed and stained for TRAP. Numbers of TRAP-positive multinucleated cells were counted. B-C, BMMs were transduced with control (pMX-IRES-EGFP) or Id2 retroviruses and cultured for the indicated days with M-CSF and TRANCE. Northern blot analysis (B) and Western blot analysis (C) were performed to assess the expression of the indicated genes. (D) BMMs derived from WT or Id2 KO mice were incubated for 3 days with M-CSF and various concentrations of TRANCE. Cultured cells were fixed and stained for TRAP. Numbers of TRAP(+) MNCs were counted.

Next, we investigated whether overexpression of Id2 in BMMs could regulate gene expression of NFATc1 and OSCAR, which are important modulators for osteoclastogenesis. TRANCE stimulation in BMMs increased the expression of NFATc1 and OSCAR in mRNA and protein levels (Figure 2B and 2C). Compared to the control, exogenous overexpression of Id2 gene attenuated the expression of NFATc1 as well as of OSCAR during TRANCE-mediated osteoclastogenesis. However, the expression of Mitf was not affected by Id2 overexpression (Figure 2B). These results indicate that Ids can regulate the gene expression of OSCAR as well as of NFATc1.

Since Ids act as negative regulators for osteoclastogenesis, we investigated their physiological role in osteoclastogenesis. BMMs from WT or Id2 KO mice were cultured with M-CSF and TRANCE. As expected, TRANCE induced osteoclast formation in a dose-dependent manner in WT (Figure 2D). Intriguingly, the number of TRAP(+) MNCs was higher in Id2 KO compared to WT. These results suggest that Ids may have a role in osteoclastogenesis as negative regulators.

It is believed that macrophages, osteoclasts, and DCs originate from the same precursors. Recently, we showed that BMMs have phagocytic activity, characteristic of macrophages, but that their phagocytic activity was abolished by stimulation with M-CSF and TRANCE *in vitro* (Takami, Kim, Rho, et al. 2002). Since the overexpression of Id genes inhibited osteoclastogenesis, we reasoned that Id gene-infected BMMs might retain phagocytic activity. When we cultured retrovirally-infected BMMs for 2 days with M-CSF and TRANCE, BMMs overexpressing Id genes did retain their phagocytic activity, whereas control vector-infected BMMs lost their phagocytic activity as a result of stimulation with M-CSF and TRANCE. When BMMs were cultured with M-CSF alone, neither overexpression of Id genes nor pMX control vector inhibited phagocytic activity (Figure 3). Previously, we demonstrated that BMMs could also become

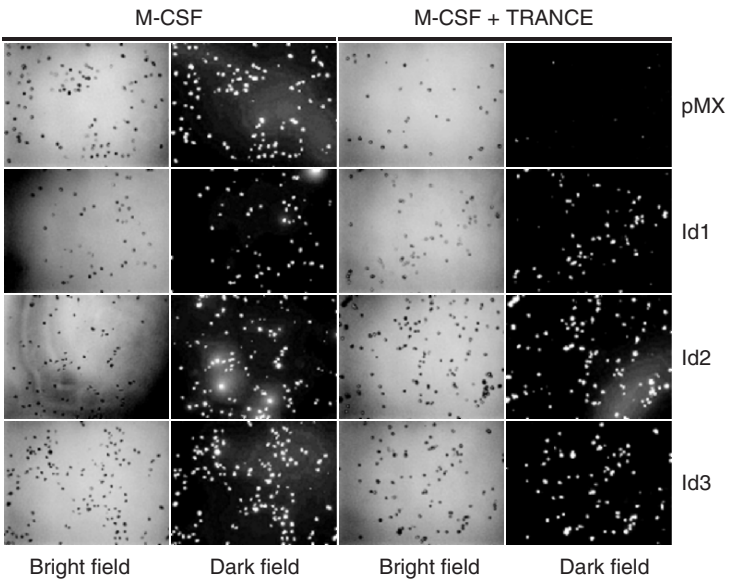

Figure 3. Overexpression of Id genes in BMMs does not affect phagocytosis. BMMs were transduced with pMX-IRES-EGFP (control), Id1, Id2, or Id3 retrovirus and cultured for 2 days with M-CSF alone or M-CSF and TRANCE. Cultured cells were incubated with Fluorescein-conjugated zymosan particles for 1 h and washed with PBS. Cells were fixed and observed with UV illumination under a microscope. Fluorescein-conjugated zymosan particles incorporated by the cells appear as bright dots in the dark field.

DCs through stimulation with GM-CSF *in vitro*. (Takami, Kim, Rho, et al. 2002) Therefore, we tested whether overexpression of Id genes affected the differentiation of BMMs into DCs. After retrovirally-infected BMMs were cultured with GM-CSF for 4 days, cells were harvested and stained for DC markers, including CD11b, CD11c, CD86, and I-A^b. Overexpression of the Id genes did not affect DC differentiation (data not shown). These data suggest that downregulation of Id genes may play a role in determining destiny of common precursors toward osteoclasts rather than macrophages or DCs.

4. THE REGULATORY MECHANISM OF OSCAR EXPRESSION BY IDS

Mitf, a bHLH transcription factor, plays an important role in osteoclast differentiation. Since HLH transcription factors can bind to bHLH proteins and inhibit transactivation of target genes, we tested whether Id proteins could bind Mitf. The direct association between Mitf and Id proteins was demonstrated using an *in vitro* pull-down assay. A Flag-tagged Mitf construct was transfected into 293T cells and Flag-Mitf protein was immobilized on anti-Flag M2 agarose beads. Beads were incubated with ^{35}S-labeled Id1, Id2, or Id3 protein and subjected to SDS-PAGE and autoradiography. Id proteins bound to Flag-Mitf but not to the control vector (Figure 4A). To demonstrate the interaction between Mitf and Id proteins in mammalian

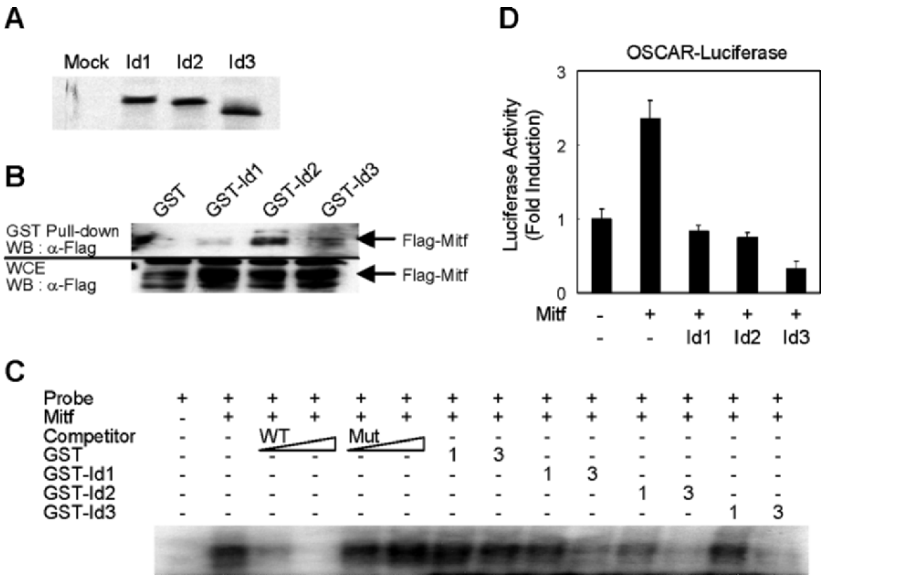

Figure 4. Ids downregulate OSCAR gene expression through interaction with Mitf. (A) ^{35}S-labeled mock, Id1, Id2, or Id3 protein was incubated with Flag-Mitf immobilized on M2-Sepharose beads, respectively. After washing, retained ^{35}S-labeled mock, Id1, Id2, or Id3 protein was examined by SDS-PAGE and autoradiography. (B) 293T cells were cotransfected with GST, GST-Id1, GST-Id2, or GST-Id3 plasmid together with Flag-Mitf plasmid. Cell lysates were immunoprecipitated with Flag-Mitf immobilized on M2-Sepharose beads. Immunoprecipitated samples (*upper panel*) and whole cell extracts (*lower panel*) were subjected to western blot analysis, respectively. (C) Mitf lysate and probe from oligonucleotide spanning E-box 2 of OSCAR promoter were used for EMSA. Specific binding was determined by cold competition using unlabeled wild- or mutant-type probe at 10- and 100-fold molar excess concentrations (lane 3–6). Mitf lysate and probe were incubated with indicated amounts (1–3μg) of GST, GST-Id1, GST-Id2, or GST-Id3 (lane 7-14). (D) 293T cells were cotransfected with OSCAR luciferase reporter and Mitf together with plasmid expressing Id1, Id2, or Id3.

cells, we performed an immunoprecipitation assay. 293T cells were cotransfected with GST, GST-Id1, GST-Id2, or GST-Id3 together with Flag-Mitf, and cell lysates were immunoprecipitated with glutathione beads. Beads were subjected to SDS-PAGE and Western blotting by anti-Flag antibody. Flag-Mitf bound to GST-Id proteins but not to GST alone (Figure 4B). These data demonstrate that Id proteins interact with Mitf in mammalian cells.

To determine whether Id proteins can modulate the binding of Mitf to E-box of OSCAR promoter, EMSA was performed in the presence of GST-Id fusion proteins. When the purified GST-Id fusion proteins were added to the reaction mixture, we observed a significant, dose-dependent decrease of Mitf binding to labeled probe (E box 2 of OSCAR promoter) caused by increasing doses of GST-Id proteins, but not by GST alone (Figure 4C). This result suggests that Id proteins decrease Mitf binding to E-box of OSCAR promoter by association with Mitf.

We have previously shown that Mitf induces expression of OSCAR, which is a modulator of osteoclastogenesis (So, Rho, Jeong, et al. 2003). Since Id proteins could bind Mitf *in vitro*, we tested whether overexpression of Ids could inhibit transactivation of the OSCAR promoter by Mitf. When 293T cells were cotransfected with a luciferase reporter plasmid containing OSCAR 1.7 Kb promoter together with Mitf vector, Mitf induced an approximately 2.5-fold increase in OSCAR promoter activity compared with control (Figure 4D). Induction of OSCAR promoter activity by Mitf was decreased by Id expression. These results suggest that Id proteins interact with Mitf and inhibit Mitf transactivation of the OSCAR gene.

5. DISCUSSION

In this study, we present evidence that Ids are responsible for the negative regulation of TRANCE-induced osteoclast differentiation, and that TRANCE promotes osteoclastogenesis, in part, by downregulating these repressors. Various transcription factors, including Mitf, PU.1, and NFATc1, are important for osteoclast differentiation. It has been shown that PU.1 is required for the correct development of both myeloid and lymphoid lineages, including macrophages, osteoclasts, DCs, neutrophils, and mast cells (Anderson, Smith, Conners, et al. 1998; McKercher, Torbett, Anderson, et al. 1996; Scott, Simon, Anastasi, et al. 1994; Tondravi, McKercher, Anderson, et al. 1997). Osteoclast differentiation fails at an early stage in PU.1-deficient mice. Mutation of Mitf results in multiple phenotypes including osteopetrosis, lack of pigment, deafness, and small eyes (Moore 1995). Although the expression of Mitf and PU.1 is not restricted to osteoclast precursor cells, only these monocytes/macrophage precursors can differentiate into mature osteoclasts via treatment with M-CSF and TRANCE. This implies that the other molecules might be involved in osteoclastogenesis.

Here, we propose a possible schematic model of OSCAR gene regulation in osteoclastogenesis based on our results and on previous studies (Figure 5). Id proteins, as well as Mitf and PU.1, are abundantly expressed in the committed precursors. Because Id proteins can directly bind to Mitf and inhibit Mitf binding to E-box of OSCAR promoter, OSCAR gene expression may be reduced to a level where it can no longer drive osteoclast formation. When TRANCE binds to its cognate receptor RANK, it induces activation of Mitf by phosphorylation of serine residues via the MKK6/p38 MAP kinase signaling cascade (Mansky, Sankar, Han, et al. 2002) and by downregulating expression of Id genes within a few hours. Subsequently, downregulation of Id genes allows Mitf to bind to its cognate E-box site, cooperating with PU.1 to induce the expression of target genes including OSCAR, TRAP, and cathepsin K (So, Rho, Jeong, et al. 2003; Luchin,

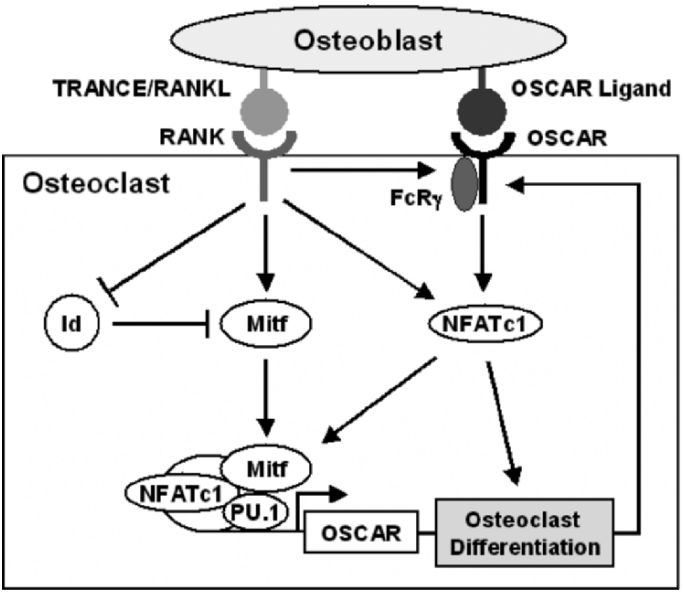

Figure 5. A schematic model of OSCAR gene regulation via transcription factors during osteoclastogenesis. Ids, Mitf and PU.1 are abundantly expressed in osteoclast precursors. In precursors, Ids interact with Mitf, therefore, inhibit its transactivation of OSCAR. TRANCE/RANK signaling activates transcription factors such as Mitf and NFATc1, and also down-regulates the expression of Id genes, subsequently leading to the upregulation of OSCAR gene during osteoclastogenesis. OSCAR and NFATc1 show positive feedback regulation.

Suchting, Merson et al. 2001; Matsumoto, Kogawa, Wada, et al. 2004). Later, TRANCE also activates NFATc1 through MKK6/p38 allowing NFATc1 to induce its target genes in a synergistic manner with other transcription factors including Mitf and PU.1 (Matsumoto, Kogawa, Wada, et al. 2004; Kim, Kim, Lee, et al. 2005). At a late stage, a positive feedback circuit involving TRANCE, NFATc1, and OSCAR induces the efficient differentiation of osteoclasts (Kim, Kim, Lee, et al. 2005; Koga, Inui, Inoue, et al. 2004; Kim, Sato, Asagiri, et al. 2005).

In this study, we observed that Id proteins directly interacted with Mitf protein and that the binding of Mitf to E-box of OSCAR promoter was inhibited by GST-Id proteins. In addition, Id genes decreased Mitf transactivation of the OSCAR gene. Furthermore, overexpression of Id2 gene in BMMs attenuated the induction of OSCAR gene, as well as osteoclastogenesis mediated by TRANCE. These results suggest that Id proteins can associate directly with Mitf and inhibit its role in osteoclastogenesis. The expression of NFATc1 was also attenuated by Id2 overexpression. Although AP-1 induces the expression of NFATc1 (Matsuo, Galson, Zhao, et al. 2004), Ids did not affect transactivation of c-fos on NFATc1 gene in transient transfection experiments (data not shown). Although the underlying mechanism of the inhibitory effect of Id2 on NFATc1 expression is not clear, one possible mechanism is that down-regulation of OSCAR by Id2 may cause the impairment of a positive feedback regulation between OSCAR and NFATc1 (Kim, Kim, Lee, et al. 2005; Kim, Sato, Asagiri, et al. 2005). However, the possibility remains that Id proteins act as negative regulators of osteoclast differentiation via interaction with other molecules.

Macrophages, osteoclasts, and DCs are thought to originate from common precursors. Since Id genes were expressed in macrophage/monocyte lineage cells, we examined whether

overexpression of Id genes affected the differentiation of BMMs into each of these cell types. Interestingly, ectopic overexpression of Id genes in BMMs inhibited osteoclastogenesis, but not phagocytic activity or differentiation into DCs. Recently, it has been shown that a HLH transcription factor Id2 is upregulated during DC development *in vitro* and crucial for the development of distinct DC subsets *in vivo* (Hacker, Kirsch, Ju, et al. 2003). These observations imply that the different expression level of Id genes in common precursors can affect cell fate upon stimulation with differentiation factors such as TRANCE, M-CSF, and GM-CSF.

6. CONCLUSION

This study demonstrates that TRANCE-mediated downregulation of Ids relieves their inhibitory effect on Mitf and subsequently on OSCAR gene expression, thereby underscoring an important role for Ids in osteoclast differentiation, and that Ids may have a physiological role in TRANCE-mediated osteoclastogenesis. To our knowledge, Ids are the first repressors in osteoclasts that are downmodulated by TRANCE. Hence, our work reveals both an additional layer of negative regulation, as well as the mechanism whereby TRANCE signaling overcomes it, allowing osteoclastogenesis to proceed. Further study of the detailed mechanism of transcription factor gene regulation will allow for a clearer understanding of the true nature of Id genes, their functions in osteoclast formation, and their utility as molecular targets for therapeutics addressing bone diseases including osteoporosis.

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CHAPTER 4
FUNCTIONAL GENETIC AND GENOMIC ANALYSIS OF MODELED ARTHRITIS

Eleni Douni, Maria Armaka, Dimitris L. Kontoyiannis, and George Kollias

1. INTRODUCTION

Rheumatoid Arthritis (RA) is a chronic inflammatory disease, where pathological interactions between synovial fibroblasts, inflammatory infiltrates and osteoclasts mediate destruction of the inflamed joint. RA and other inflammatory bone diseases illustrate the close link between bone biology and immunology. There is growing evidence that certain molecules regulate both the skeletal and immune systems. Among these factors, tumor necrosis factor (TNF) has received great attention because of its position at the apex of the pro-inflammatory cytokine cascade, which is responsible for the development of inflammatory diseases such as RA and inflammatory bowel disease (IBD). TNF is expressed at high levels in inflamed synovium from RA patients (Saxne, Palladino, Heinegard, et al. 1988; Brennan, Chantry, Jackson, et al. 1989; Firestein, Alvaro-Gracia, and Maki 1990; Feldmann, Brennan, and Maini 1996) where it has pleiotropic functions such as the induction of joint inflammation, the proliferation of synovial fibroblasts (SFs) (Butler, Piccoli, Hart, et al. 1988), the destruction of cartilage by induction of collagenase (Dayer, Beutler, and Cerami 1985) and inhibition of proteoglycan synthesis by articular chondrocytes (Saklatvala 1986). In addition, TNF has a central role in bone pathophysiology by stimulating osteoclastogenesis and bone resorption, while stimultaneously inhibiting the function of bone-forming osteoblasts (Nanes 2003). Experimental studies in animal models of arthritis offer critical information regarding the specific function of TNF and its receptors, TNFRI and TNFRII (Douni, Akassoglou, Alexopoulou, et al. 1995). Particularly, transgenic mice overexpressing human TNF developed an erosive inflammatory arthritis closely resembling human RA (Keffer, Probert, Cazlaris, et al. 1991) and foreshadowed the remarkable effectiveness of anti-TNF therapy in RA (Elliott, Maini, Feldmann, et al. 1993). In this review, we describe the cellular and molecular pathways involved in the pathogenesis of TNF-mediated arthritis in animal models generated by our research group in the past.

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2. PROINFLAMMATORY FUNCTIONS OF TNF OVEREXPRESSION

TNF expression is tightly regulated post-transcriptionally through the AU-rich element (ARE) in the 3' untranslated region (UTR) of the mRNA. In resting conditions, the ARE mediates rapid degradation of TNF transcripts. TNF ARE binding proteins such as tristetraprolin (TTP) and T-cell intracellular antigen-1 (TIA-1), regulate TNF overexpression and may function as arthritis suppressors (Taylor, Carballo, Lee, et al. 1996; Phillips, Kedersha, Shen, et al. 2004). TTP inhibits TNF production from macrophages by destabilizing its mRNA (Carballo, Lai, and Blackshear 1998), while TIA-1 represses the expression of TNF by inhibiting its translation (Firestein, Alvaro-Gracia, and Maki 1990; Piecyk, Wax, Beck, et al. 2000). The significance of these interactions in inflammation has been exemplified in mouse systems with deficiencies in the TNF 3'ARE or the RNA binding proteins TTP and TIA-1, which are predisposed to chronic inflammatory pathologies (Taylor, Carballo, Lee, et al. 1996; Kontoyiannis, Pasparakis, Pizarro, et al. 1999; Piecyk, Wax, Beck, et al. 2000). In addition, recent evidence shows suppressed inflammatory responses in vivo by inducible overexpression of HuR, which is also an TNF RNA binding protein (Katsanou, Papadaki, Milatos, et al. 2005).

Therefore, it is rather straightforward to explain why human TNF transgenic mice (huTNFtg) generated in early '90s, in which the 3' UTR was replaced by that of the β-globin gene, over expressed TNF and as a result developed chronic, erosive, inflammatory polyarthritis with histologic lesions resembling human RA (Keffer, Probert, Cazlaris, et al. 1991). This transgenic mouse model of arthritis is an ideal tool for evaluating the efficacy of novel theupareutic agents for rheumatoid arthritis (Douni, Sfikakis, Haralambous, et al. 2004; Thwin, Douni, Aidinis, et al. 2004).

A similar form of arthritis but also Crohn's-like IBD appeared in targeted mutant mice lacking the ARE region ($TNF^{\Delta ARE}$ mutant mice), confirming the role of these elements in the maintenance of a physiological TNF response in the joint. In this animal model, the absence of TNF ARE leads to (a) increased TNF mRNA stability; (b) constitutively active translation of the TNF mRNA; (c) chronic overproduction of TNF protein; (d) permissive TNF translation in ectopic sites (e.g. in synovial fibroblasts); (e) loss of anti-inflammatory translational controls; and (f) chronic inflammatory arthritis and Crohn's-like IBD. Lack of the ARE regulatory sequences confers a state of anti-inflammatory unresponsiveness to TNF mRNA translation, allowing for sustained overexpression of TNF protein, which is apparently causative of disease (Kontoyiannis, Pasparakis, Pizarro, et al. 1999).

3. TNF SOURCE AND TARGET CELLS IN MODELED ARTHRITIS

To gain insight into the cellular basis of pathogenic TNF/TNF receptor function in TNF-modeled arthritis, we performed reciprocal bone marrow grafting experiments into lethally irradiated hosts. Wild-type, $TNF^{\Delta ARE}$, TNFR knockout and $TNF^{\Delta ARE}$/TNFR mice were used as either recipients or donors of bone marrow cells. From all combinations studied, we demonstrate that in the $TNF^{\Delta ARE}$ model, bone marrow cells constitute a cellular source of TNF, which is sufficient for the induction of arthritis. However, the development of arthritis requires the expression of TNFRI in the recipient mice, demonstrating that cellular targets of TNF function in arthritis are located exclusively in the radio-resistant compartment. When this type of experiments was performed in huTNFtg mice, arthritic phenotype was evident exclusively to the chimeric mice in which huTNF source and target (through TNFRI) was located in stromal-radioresistant compartment (M. Armaka, to be published elsewhere).

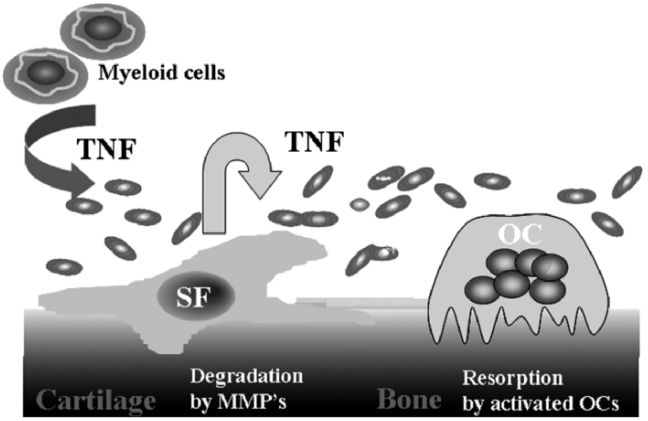

Figure 1. In TNF-modeled arthritis pathogenic TNF loads can derive either from haemopoietic (possibly cells of myeloid lineage) or stromal-radioresistant cell types (such as synovial fibroblasts, SFs). Intrinsic TNFRI signaling in radioresistant cells (SFs, osteoclasts (OC)) can initiate and perpetuate the degradation of cartilage by TNF-induced MMPs expression by SFs and bone resorption by mature OCs.

Using the reciprocal bone marrow grafting experiments, we show that development of arthritis in TNF overexpressing mice requires the expression of TNFRI in cells of the radiation-resistant compartment. However, the TNFRI requirements are independent of the pathogenic cellular sources of TNF (haemopoietic or stromal) indicating redundancy in arthritogenic TNF pools (Figure 1). Interestingly, both radiosensitive or radioresistant cell types are sufficient targets for TNF in the development of the Crohn's-like IBD in $TNF^{\Delta ARE}$ mice (Kontoyiannis, Boulougouris, Manoloukos, et al. 2002). Therefore, multiple cell types and compartments can work independently to produce TNF-dependent pathologic phenotypes such as in arthritis or IBD.

4. SIGNALS DOWNSTREAM OF TNF

The arthritogenic effects of human TNF overproduction in the huTNFtg model are mediated exclusively through the activation of TNFRI (Keffer, Probert, Cazlaris, et al. 1991), since due to species specificity, human TNF binds only to mouse TNFRI (Lewis, Tartaglia, Lee, et al. 1991). However, the presence of a human TNFRII transgene (Douni and Kollias 1998), exacerbated the progression of arthritis in the huTNFtg mouse model by increasing dramatically the infiltration of polymorphonuclear cells locally in the joint (Douni, unpublished observation). A similar dominant role for TNFRI in mediating the $TNF^{\Delta ARE}$ pathologies was revealed in such mutant mice deficient for the TNFRI (Kontoyiannis, Pasparakis, Pizarro, et al. 1999), suggesting that TNFRI consists an important target for novel therapeutic approaches in RA. On the contrary, a much more aggressive and destructive type of arthritis appeared in $TNF^{\Delta ARE}$ mice lacking TNFRII. At the histological level, such mice excibited an exacerbated synovial hyperplasia with increased numbers of polymorphonuclear infiltrates and enchanced destruction of cartilage and bone (Kontoyiannis, Pasparakis, Pizarro, et al. 1999). Progression of arthritis agrees with the previously described inhibitory role of TNFRII in osteoclastogenesis (Abu-Amer, Erdmann, Alexopoulou, et al. 2000). However, the contradictory results of

TNFRII contribution between the two TNF-mediated arthritis models could be explained by differences in underlying mechanisms.

Mitogen-activated protein kinases (MAPKs) have been associated with the pathogenesis of RA, whereas the individual contribution of the three family members, p38s, c-Jun N-terminal kinases (JNKs) and extracellular signal-related kinases (ERKs), remains largely unknown. In vivo, overexpression of TNF in transgenic mice induced activation of p38α and ERKs in the synovial membrane, whereas activation of JNKs was less pronounced and rarely observed. Activated p38α was predominantly found in synovial macrophages, whereas ERK activation was present in both synovial macrophages and fibroblasts (Gortz, Hayer, Tuerck, et al. 2005). p38s are key mediators of TNF functions, involved on TNF signaling but also on its biosynthesis through interactions with ARE. Therefore, the involvement of p38s in TNF ARE-deficient mice, are exclusively related to TNF signaling. Inhibition of p38 kinases by specific inhibitors decreased the severity of synovial inflammation and cartilage damage and protected against bone destruction caused by overexpression of TNF in transgenic mice (Zwerina, Hayer, Redlich, et al. 2006). This suggests that p38 kinases, are important signaling molecules downstream of TNF promoting both synovial inflammation and osteoclastogenesis. However, the progression of the $TNF^{\Delta ARE}$-induced pathologies were not moderated by knockout of the p38β isoform (Beardmore, Hinton, Eftychi, et al. 2005), implying that p38α seems to be the major isoform involved in the pathogenesis of inflammatory diseases. Unexpectedly, an exacerbation of TNF-induced pathologies was observed in $TNF^{\Delta ARE}$ mice lacking MK2, a kinase downstream of p38α and β (Kontoyiannis, Boulougouris, Manoloukos, et al. 2002). On the other hand, JNK1 activation is not required for chronic destructive arthritis in the huTNFtg model of arthritis (Koller, Hayer, Redlich, et al. 2005).

Apart from TNF, two other inflammatory cytokines are involved in the pathogenesis of RA, interleukin-1 (IL-1) and interleukin-6 (IL-6). Treatment of arthritic huTNFtg mice with antibodies against the type I IL-1 receptor leads to neutralization of arthritis, supporting the idea that a functional hierarchy exists in which the IL-1 receptor acts in series and downstream of TNF to effect TNF-mediated arthritogenic responses (Probert, Plows, Kontogeorgos, et al. 1995). However, the arthritic pathology in huTNF transgenic mice is IL-6 independent (Alonzi, Fattori, Lazzaro, et al. 1998).

5. CELLULAR INTERACTIONS IN ARTHRITIS

Upon initiation of RA, the synovial membrane becomes hyperplastic due to the accumulation of fibroblasts and cells of haemopoietic origin. This alteration is followed by the destruction of neighboring structures, such as articular cartilage and bone due to invasive properties of synovial tissue which is based on a tight interplay between synovial fibroblasts, lymphocytes and osteoclasts. The involvement of immune cells is a hallmark of autoimmune disorders. However, the concept that RA is a T-cell-dependent disease has been strongly challenged during the past years. The minimal, if any, role of adaptive immunity in the development of TNF-mediated arthritis has been confirmed in huTNFtg and $TNF^{\Delta ARE}$ mutant mice where the course of disease is not affected by the absence of mature T and B cells (Douni, Akassoglou, Alexopoulou, et al. 1995; Kontoyiannis, Pasparakis, Pizarro, et al. 1999). This is in agreement with transplantation studies showing destruction of human cartilage by RA derived synoviocytes engrafted in the kidney capsule of SCID mice (Geiler, Kriegsmann, Keyszer, et al. 1994).

Synovial fibroblasts (SFs) derive from the mesenchymal lineage, and their primary function is to provide mechanical strength to tissues by secreting a supporting framework of extracellular

matrix. There is growing evidence that activation of SFs is an early step in the development of RA. Recent data support the involvement of Toll-like receptors (TLRs), which are key recognition structures of the innate immune system, at initial stages of synovial activation (Brentano, Kyburz, Schorr, et al. 2005). Synovial hyperplasia, which is definitely one of the most prominent characteristics of RA, could result from increased proliferation of RA SFs either spontaneously or in response to cytokines, such as TNF. Increased expression of c-myc, c-jun and nuclear factor-κB (NF-κB) indicates that hyperproliferation may indeed occur (Kontoyiannis and Kollias 2000; Pap, Móller-Ladner, Gay, et al. 2000). An alternative explanation to hyperproliferation is defective apoptosis. Apoptotic signals provided by molecules such as PTEN (Pap, Franz, Hummel, et al. 2000), c-myc (Pap, Nawrath, Heinrich, et al. 2004), p16 (Taniguchi, Kohsaka, Inoue, et al. 1999) and SENTRIN (Franz, Pap, Hummel, et al. 2000) have been reported as modified in RA SFs, and these cells show reduced apoptosis in situ. Our studies show that SFs from arthritic TNF overexpressing mice do have increased proliferation but also increased tolerance to apoptosis in comparison to wild-type SFs (unpublished observation).

Accumulated evidence suggesting that arthritic SFs exhibit characteristic of transformed cells led to the working hypothesis that the arthritic synovium is a locally invasive tumour (Firestein and Zvaifler 2002). High-throughput expression profiling has recently revealed cytoskeletal rearrangements in arthritic SFs derived from both TNF-mediated arthritis models, huTNFtg and $TNF^{\Delta ARE}$ mice. Detection of pronounced stress fibers in arthritic SFs confirms the expression profiling results. Differences in the actin cytoskeleton reflect altered extracellular matrix attachment properties. Indeed, arthritic SFs adhere to different proteins of the ECM such as fibronectin, vitronectin, laminin and collagen, with increased affinity in vitro (Aidinis, Carninci, Armaka, et al. 2005). Therefore, it seems that one possible pathogenic mechanism in RA is the promotion of actin polymerization and rearrangement of the actin cytoskeleton. The rearranged cytoskeleton in arthritic SFs reinforces the concept of a transformed-like character of the SFs and opens up new directions in the pharmacological treatment of RA.

In response to extracellular factors, SFs themselves release a plethora of effector cytokines and chemokines that mediate the interaction with neighbouring inflammatory and endothelial cells, promoting matrix degradation and progressive destruction of articular cartilage and bone. Cytokines and chemokines secreted by fibroblasts are an important link between the innate and acquired immune responses and play a crucial role in determining the nature and magnitude of the inflammatory infiltrate (Buckley, Pilling, Lord, et al. 2001). SFs either from huTNFtg or $TNF^{\Delta ARE}$ mice constitutively produce high basal levels of soluble TNF while SFs from wild-type mice do not secrete TNF even upon LPS stimulation (Kontoyiannis, Pasparakis, Pizarro, et al. 1999). SFs instigate leukocyte attraction and homing through the expression of chemokines like MIP-1, RANTES, interleukin-8 and interleukin-16 (Smith, Smith, Blieden, et al. 1997; Szekanecz, Szucs, Szanto, et al. 2006). SFs can also support myeloid and lymphoid cell growth via the secretion of various colony stimulating factors, as well as their own growth via the production of platelet derived growth factor (PDGF) (Butler, Leizer, and Hamilton 1989).

Various direct and indirect mechanisms contribute to the progressive destruction of articular cartilage and the adjacent bone. Destruction of the articular cartilage is mainly mediated by attachment of SFs to the underlying cartilage by upregulation of cellular adhesion molecules and release of matrix-degrading enzymes such as matrix metalloproteinases (MMPs) and cathepsins (Muller-Ladner, Kriegsmann, Franklin, et al. 1996). This process seems to be autonomously regulated since it is now clear that it does not require immune effector cells (Geiler, Kriegsmann, Keyszer, et al. 1994). SFs are also involved in the differentiation of myeloid cells into osteoclasts through upregulation of receptor activator of NF-κB ligand (RANK-L) (Takayanagi, Iizuka, Juji, et al. 2000; Nakano, Okada, Saito, et al. 2004).

6. BONE EROSION IN ARTHRITIS

Bone remodeling is a dynamic process which is accomplished by a tight interplay between bone-forming cells, osteoblasts, and bone resorbing cells, osteoclasts (Karsenty 2003). This turnover ensures the plasticity of the bone while misbalance between osteoblast and osteoclast activity either leads to increased bone mass (osteopetrosis) or loss of bone mass (osteoporosis), which is a typical aspect of RA (Woolf 1991). Osteoclasts are derived from myeloid lineage cells which are generated in bone marrow and released in the periphery through the bloodstream. Numerous osteoclast precursor cells (OCPs) accumulate in the synovium of animals or patients with RA, either from "outside in" i.e. from blood to the pannus-bone interface or "inside out" i.e. from bone marrow to the subchondral bone. HuTNFtg mice have increased numbers of OCPs in spleen and blood as well as enhanced numbers of mature osteoclasts, interposed between the bone surfaces and the "erosive front" of the synovium (Li, Schwarz, O'Keefe, et al. 2004b). Recent evidence shows that TNF stimulates bone marrow OCP genesis by upregulation of c-Fms expression which further results to OCP proliferation and differentiation in response to macrophage colony stimulating factor (M-CSF) (Yao, Li, Zhang, et al. 2006). Therefore, the first step of TNF-induced osteoclastogenesis is at the level of OCP formation in the bone marrow.

OCPs differentiate into mature osteoclasts in response to osteoclastogenic cytokines such as M-CSF, RANK-L, and TNF which are produced locally in the inflamed synovium (Goldring 2003). It has been previously shown that TNF lowers the threshold of RANK-L requirements for osteoclastogenesis, in vitro. However, it seems that TNF-induced osteoclastogenesis requires at least constitutive levels of RANK-L (Lam, Takeshita, Barker, et al. 2000). This dependency of TNF-mediated osteoclastogenesis on attendant RANK-L is underscored by the absence of osteoclast formation in arthritic huTNFtg mice in which the RANK-L receptor has been deleted (Li, Schwarz, O'Keefe, et al. 2004a). Recent evidence suggest that TNF-induced osteoclastogenesis involves an increase of RANK-L synthesis by bone marrow stromal cells through IL-1/IL-1RI enhanced expression (Wei, Kitaura, Zhou, et al. 2005). TNF stimulates RANK-L production by stromal cells (Hofbauer, Lacey, Dunstan, et al. 1999), T lymphocytes (Cenci, Weitzmann, Roggia, et al. 2000), B lymphocytes (Kanematsu, Sato, Takai, et al. 2000) and endothelial cells (Collin-Osdoby, Rothe, Anderson, et al. 2001) while stimulates M-CSF production by stromal cells (Srivastava, Toraldo, Weitzmann, et al. 2001). Severe osteopetrosis due to complete absence of osteoclasts, developed in mice deficient either for M-CSF (Wiktor-Jedrzejczak, Bartocci, Ferrante, et al. 1990), or RANK-L/RANK (Dougall, Glaccum, Charrier, et al. 1999; Kim, Odgren, Kim, et al. 2000) but not in TNF knockout mice (Pasparakis, Alexopoulou, Episkopou, et al. 1996). However, OPCs from RANK-L or RANK null mice can still differentiate into osteoclasts in vitro, upon stimulation with TNF in the presence of cofactors such as TGF-β (Kim, Kadono, Takami, et al. 2005). These results show that RANK-L is a major regulator of osteoclast differentiation while an alternative pathway of osteoclast differentiation may also exists in vivo.

Mature osteoclasts are essential effector cells for normal bone remodeling and pathologic bone loss observed in various erosive diseases such as RA. Knockout mice deficient in genes essential for osteoclastogenesis develop osteopetrosis because of the accumulation of unresorbed bone matrix within the bone marrow cavity (Tolar, Teitelbaum, and Orchard 2004). These mice are completely resistant to bone destruction in arthritogenic conditions such as in the huTNFtg mice (Redlich, Hayer, Ricci, et al. 2002). Similarly, blockade of bone resorption by osteoclast-targeted therapies has been shown to preserve systemic bone mass effectively in huTNFtg mice (Schett, Redlich, Hayer, et al. 2003). These results clearly show that osteoclasts are essential for TNF-mediated bone loss whereas intervening in RANKL:RANK/osteoprotegerin signaling to preserve skeletal integrity in inflammatory arthritis appears to be a promising approach.

The current model of local bone erosion in chronic arthritis can be summarized to the following steps (Schett, Hayer, Zwerina, et al. 2005); i) formation of a pro-osteoclastogenic microenvironment where OCLs enter the synovial membrane, ii) induction of RANK-L expression locally in the synovial tissue i.e. by activated T lymphocytes and SFs, iii) binding of RANK-L to RANK on OCLs and initiation of osteoclastogenic signaling cascade and iv) resorption of subchondral bone by the activated osteoclast, which involves the generation of an acidic pH that solubilizes calcium and the secretion of enzymes that remove the bone matrix. This final step leads to bone erosion as it is observed histologically at the interface between the pannus and the bone.

7. FUTURE CONSIDERATIONS

Complementary to 'gene driven' approaches involving manipulation of TNF either by transgenesis or targeted mutagenesis, we also employ 'phenotype driven' approaches in which new genes will be identified by recovering mouse mutants with novel phenotypes. Genome-wide, random mutagenesis with the ethylating mutagen N-ethyl-N-nitrosourea (ENU) has become an attractive method to track the role of virtually any gene in a particular phenotype. In particular, ENU mutagenesis of disease sensitized animals offers unique opportunities to discover gene functions directly associated with prevention or therapy of diseases. We have thus initiated a programme of sensitized ENU mutagenesis screen applied on our established $TNF^{\Delta ARE}$ model of RA and IBD, to identify modifier gene candidates associated with development of these diseases. By using specific phenotypic screens for arthritis and IBD, the individual progeny that display disease attenuation are selected and propagated for further functional characterization and genetic mapping analysis. Once identified these novel gene functions may constitute validated pharmaceutical targets for the treatment of chronic inflammatory disease such as arthritis and IBD.

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CHAPTER 5
DEXAMETHSONE SUPPRESSES BONE FORMATION VIA THE OSTEOCLAST

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Glucocorticoids are central to treating inflammatory and immune disorders. These steroids, however, profoundly impact the skeleton, particularly when administered for prolonged periods. In fact, high-dose glucocorticoid therapy is almost universally associated with bone loss, prompting among the most common forms of crippling osteoporosis. Despite the frequency and severity of glucocorticoid-induced osteoporosis, its treatment is less than satisfactory, suggesting that its pathogenesis is incompletely understood.

Net bone mass represents the relative activities of osteoblasts and osteoclasts and there is little question that glucocorticoids suppress the bone-forming cells, in vivo, via a process involving accelerated apoptosis (Weinstein 2001; Weinstein, Jilka, Parfitt, et al. 1998). Surprisingly, however, addition of glucocorticoids to cultures of osteoprogenitor cells actually increases their capacity to form mineralized bone nodules (Aubin 1999; Purpura, Aubin, and Zandstra 2004). This paradox raises the possibility that glucocorticoid suppression of bone formation, in vivo, reflects, at least in part, the steroid's targeting intermediary cells, which in turn inhibit the osteoblast.

Bone remodeling is an ever-occuring event in mammals which is characterized by tethering of osteoclast and osteoblast function. The process is initiated by osteoclasts (OCs) resorbing a packet of bone, which in turn leads to osteoblasts being recruited to the site of resorption. This process establishes that osteoclastic bone resorption, in some manner, promotes osteoblastic bone formation at the same location. Consequently, pathologically or pharmacologically inhibited resorption eventuates in arrested osteoblast activity.

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Bone resorption reflects the sum of OC recruitment and death and the rate at which the average cell degrades matrix. Each is controversial regarding glucocorticoid therapy, as the drugs are variously perceived to suppress (Hirayama, Sabokbar, and Athanasou 2002) or enhance (Manolagas and Weinstein 1999) generation of these cells. In particular, there is little evidence that the steroids impact the resorptive machinery of the mature polykaryon.

Glucocorticoids, which arrest osteoblast differentiation also stimulate RANKL (Hofbauer, Gori, Riggs, et al. 1999) and M-CSF (Rubin, Biskobing, Jadhav, et al. 1998) expression, which, in conjunction with the steroid's anti-apoptotic effect on the mature bone resorptive cell (Kim, Zhao, Kitaura, et al. 2006; Weinstein, Chen, Powers, et al. 2002) should increase bone degradative activity. It is, therefore, curious that there is little evidence that accelerated bone resorption contributes to the profound osteoporosis complicating prolonged glucocorticoid therapy. In fact, the histologic appearance of bones of patients, so treated, indicates that bone resorption, as well as formation, are diminished (Dempster 1989; Prummel, Wiersinga, Lips, et al. 1991). On the other hand, the conundrum of glucocorticoid-inhibited bone resorption, in the face of stimulated RANKL and M-CSF expression and prolonged OC lifespan, may reflect direct suppression by the steroid of the cell's resorptive machinery. We, therefore, undertook a series of studies to determine whether glucocorticoids directly talk to mature OCs and, if so, does this translate into suppression of bone forming activity (Kim, Zhao, Kitaura, et al. 2006).

These experiments involved generation of mice in which the glucocorticoid receptor is conditionally deleted in OC-lineage cells. This was accomplished by mating heterozygous mice harboring a floxed exon 2 allele of the glucocorticoid receptor to *LysM-Cre* transgenic mice. The product of this mating served as negative control for all experiments.

Our first exercise was to determine if glucocorticoids impact proliferation of OC precursor cells. In fact, proliferation of bone marrow macrophages (BMMs) incubated with dexamethasone (DEX) progressively decreases in a dose-dependent fashion. On the other hand, when cells are exposed to RANKL or TNF for 3 days, circumstances which promote osteoclast differentiation, glucocorticoids failed to impact their proliferation. Establishing specificity, IL-1 does not arrest the anti-proliferative effects of the steroid. Confirming the data of Weinstein et al. (Weinstein, Chen, Powers, et al. 2002), we also find that glucocorticoids inhibit OC apoptosis and require only the last 24 hours of a 5-day culture period to do so. On the other hand, DEX fails to impact differentiation as determined by the OC markers TRAP, MMP9 and cathepsin K.

We next turned to the effects of DEX on mature OCs. We find that addition of the steroid to osteoclastogenic cultures progressively inhibits the capacity of the mature OC to spread in cultures, again in a dose-dependent manner. This observation is indicative of a cytoskeletal effect of the steroid on the bone resorptive cell. In fact, the presence of DEX in concentrations of 10nm, virtually arrests all OC spreading in culture. Moreover, significant inhibition of spreading occurs at concentrations of the steroid as low as 1nm. Similar to its effect on apoptosis, the presence of DEX for the last day of a 5-day culture period completely blocks OC spreading. Thus, whereas glucocorticoids have no effect on OC differentiation, they arrest cytoskelet al organization of mature resorptive cells.

The OC enjoys a unique cytoskeleton in that it forms actin rings upon contact with bone (Teitelbaum 2000). The actin ring separates the bone resorptive microenvironment from the general extracellular space and is central for optimal bone resorptive activity. In keeping with the cell's failure to spread, glucocorticoids specifically inhibit M-CSF induced actin ring formation in OCs. This phenomenon is reflected by the failure of DEX-treated OCs to undergo bone resorptive activity when placed dentin slices. Confirming specificity, the steroid

does not disrupt the cytoskeleton of cells treated with RANKL, TNF or IL-1 alone. Thus, glucocorticoids specifically arrest M-CSF driven cytoskelet al organization of OCs.

The OC cytoskeleton is regulated by the Rho family of GTPases, including RhoA (Chellaiah, Soga, Swanson, et al. 2000) and Rac (Korhonen, Zhao, Faccio, et al. 2005). To determine if DEX impacts activation of these small GTPases, we treated the cells with a steroid for 16 hours and then exposed the cells to M-CSF. In fact, DEX completely arrests M-CSF induction of RhoA and Rac activity.

Small GTPases such as Rac, transit from their inactive GDP-bound form to their active GTP bound configuration under the aegis of guanine nucleotide exchange factors (GEFs). Vav3 is an OC-selective GEF, which activates Rac (Faccio, Teitelbaum, Fujikawa, et al. 2005). In keeping with the inhibitory effect of DEX on Rac activation, the same obtains as regards Vav3. Specifically, 16 hours of glucocorticoid treatment completely arrests tyrosine phosphorylation of the GEF.

Having established that glucocorticoids inhibit OC function, in vitro, we asked if the same obtains, in vivo. To this end, we treated cells with PTH to mobilize OCs in the presence and absence of DEX. Histological sections of calvaria were stained for TRAP activity to identify OCs. While glucocorticoids do not impact OC number in the presence or absence of parathyroid hormone (PTH), the appearance of the OCs in DEX treated mice is distinctly abnormal. Mirroring their in vitro phenotype, the cells fail to spread in vivo nor do they develop the critical bone resorptive organelle, the ruffled membrane. Attesting to the failure of glucocorticoid-treated OCs to respond to a bone resorptive stimulus, PTH does not increase circulating TRAcP5b, a marker of global bone resorption, in DEX-treated mice.

The evidence presented thus far confirms that glucocorticoids directly target the OC in vitro and in vivo and in so doing arrests its capacity to organize its cytoskeleton and resorb bone. Because the bone remodeling process is characterized by a coupling of bone resorption followed by formation, we hypothesized that glucocorticoid-mediated disruption of OC function, might contribute to the drug's osteoblast-suppressive effects. To determine is such is the case, we treated WT mice and those lacking the glucocorticoid receptor in OC-lineage cells with DEX. We then assessed bone formation, histologically and biochemically. Using double tetracycline labeling, we observed decreased mineral apposition and bone formation rates in DEX-treated WT mice, but not in those lacking the glucocorticoid receptor in their OCs. Confirming global inhibition of osteoblast function, serum osteocalcin and alkaline phosphatase levels mirror the histomorphometrically-determined kinetic data.

Thus, while glucocorticoids directly suppress osteoblasts, a substantial component of their anti-bone-forming properties relates to their direct effect on OCs. At present, there is little insight into the mechanisms by which DEX-treated OCs dampen osteoblastic activity but two general hypotheses present themselves. First, inhibited OC function will lead to decreased mobilization of bone matrix-residing, osteoblast-stimulating growth factors such as TGF-β (Rickard, Sullivan, Shenker, et al. 1994). Second, glucocorticoids may also impact cytokine production by the OC, *per se*. Resolution of these issues will not only help define the pathogenesis of glucocorticoid-induced osteoporosis, but the general mechanisms of bone remodeling as well.

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CHAPTER 6
IMMUNOLOGIC REGULATION OF BONE DEVELOPMENT

Mark C. Horowitz[1] and Joseph A. Lorenzo[2]

1. ABSTRACT

A regulatory network comprised of transcription factors PU.1, Ikaros, E2A, EBF, and Pax5 control B cell fate specification and differentiation. Early B Cell Factor-1 (EBF-1) is essential for B cell fate specification while Pax5 is required for B cell development. Mice deficient in Pax5 or EBF-1 have a developmental arrest of B cell differentiation at the pro-B cell stage, which results in the absence of mature B cells. We analyzed the bone phenotype of Pax5 and EBF-1 wild-type (+/+) and homozygous mutant (−/−) mice to determine if the loss of these transcription factors regulated bone cell development.

Bones from Pax5−/− mice were strikingly osteopenic 15 days after birth, with increased numbers of osteoclasts, and decreased trabecular number. The number of osteoblasts in Pax5−/− bones and their function in vitro were not different from controls. In addition, Pax5 was not expressed by wild-type osteoblasts. To investigate the origin of the in vivo increase in osteoclasts, Pax5−/− or +/+ spleen cells were cultured with M-CSF and RANKL and multinucleated, $TRAP^+$ cells counted. Cells from Pax5−/− spleen produced 5-10 times more osteoclasts than did controls.

Tibia from EBF-1−/− mice had a striking increase in osteoblasts lining bone surfaces. Consistent with this was an increase in osteoid thickness and in the bone formation rate. This correlated with a 2-fold increase in serum osteocalcin. However, in vitro proliferation and ALP of mutant osteoblasts did not differ from control. In contrast, osteoclast number was similar in 4 week-old +/+ and −/− mice; however, at 12 weeks the number of osteoclasts was more than twice that of controls. These data correlated with an increase in bone volume at 12 weeks of age. The most striking aspect of the EBF-1−/− bones was the presence of adipocytes, which filled the marrow space. The adipocytes in the marrow were present at both 4 and 12 weeks of age. Increased fat was also seen in the liver of mutant mice. However, subcutaneous fat was almost absent in EBF-1−/− mice. Importantly, EBF-1 mRNA was expressed in wild-type osteoblasts and in adipocytes.

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Loss of EBF-1 and Pax5 causes distinct, non-overlapping bone phenotypes. It is important to understand why this network of transcription factors, which are so important for B cell development, have such striking effects on bone cell growth and development.

2. INTRODUCTION

It is clear that the immune and hematopoietic systems are closely related. In fact, immune cells arise from hematopoietic stem cells (HSC) in the bone marrow (BM). In a similar manner, the hematopoietic and skeletal systems are related. It is now clear that osteoclasts are hematopoietic in origin and are members of the macrophage lineage. However, it has only been recently appreciated that the immune and skeletal systems are also linked. Cytokines and their receptors that were thought to be expressed exclusively by immune cells have now been shown to not only be expressed by bone cells but are critical for osteoblast and osteoclast development and function. As an example, one of the ways early osteoclast precursors respond to M-CSF binding is by the expression of receptor activator of NFκB (RANK) (Hofbauer and Heufelder 2000). RANK is a member of the TNF family of receptors and is the cognate receptor for receptor activator of NFκB ligand (RANKL), which along with M-CSF, is required for osteoclast differentiation. RANK was originally identified on dendritic cells while RANKL was identified on T cells.

Osteoclast differentiation is a highly regulated process controlled by the expression of specific transcription factors and the interactions of a group of related cytokine-cytokine receptor interactions. As an example, early in osteoclast development the expression of the transcription factor PU.1 is required for further osteoclast development. PU.1 deficient mice fail to develop both macrophages and osteoclasts (Tondravi, McKercher, Anderson, et al. 1997). In addition to the requirement of PU.1 for osteoclast development, PU.1 is a member of a regulatory network of transcription factors that include PU.1, Ikaros, E2A, EBF-1, and Pax5 that control B cell fate specification and differentiation. Importantly, these transcription factors, in addition to their regulation of B cell differentiation, have important and apparently non-overlapping effects on bone cell development. This is another example of the relationship between the skeletal and immune systems and that many more unappreciated or unidentified examples await to be revealed. The study of the relationship between the skeletal and immune systems is now known as osteoimmunology.

3. EBF

EBF-1 is the founding member of small multigene family of helix-loop-helix (bHLH) proteins that are evolutionarily conserved with defined roles in cellular differentiation and function. These genes are involved in such diverse roles as sex determination in Drosophila and B-lymphopoiesis in mice (Campos-Ortega 1998; Dambly-Chaudiere and Vervoort 1998; O'Riordan and Grosschedl 1999). This motif was originally identified as a novel type of HLH protein with a dimerization domain containing two helices with homology to the second helix of the classical bHLH protein dimerization domain, but with a different type of DBA binding domain (Hagman, Belinger, Travis, et al. 1993). This factor was cloned from both S. cerevisiae in experiments aimed at identifying the olfactory-restricted olfactory marker protein-1 (OMP-1) promoter (Want and Reed 1993; Wang, Tsai, and Reed 2002) and by biochemical purification of a factor interacting with the B lymphocyte restricted mb-1 promoter (Hagman, Belanger, Travis, et al. 1993). It was named Olf-1, or Early B Cell Factor (EBF), which in turn led to the

designation of the factor as O/E-1. Mice express at least three more members of this family, EBF-2 (mMot1/O/E-3), EBF-3 (O/E2) (Walther, Guenet, Simon, et al. 1991; Dambly-Chaudiere and Vervoort, et al. 1998) and O/E-4 (Garel, Marin, Mattei, et al. 1997; Wang, Betz, and Reed 2002). Isolation of the O/E homologue Collier from Drosophila indicated the existence of a new family of evolutionarily conserved proteins [Collier/Olf/EBF (COE)].

O/E proteins play an important role in embryonic development. As an example, antisense Collier RNA results in abnormal head development in Drosophila (Crozatier, Valle, Dubois, et al. 1996). Interestingly, OAZ is an O/E interacting protein involved in BMP signaling (Hata, Seoane, Lagna, et al. 2000). OAZ interacts with Smad1 where Smads and O/E proteins compete for OAZ. Thus, it may be that O/E proteins regulate BMP signaling.

Because EBF-1 expression is critical for B cell development, mice deficient in EBF-1 lack all B cells except those at the earliest stages of development (cells expressing B220 and high levels of CD43, pro-B cells) (Hardy, Carmack, Shinton, et al. 1991; Lin and Grosschedl 1995). The absence of D-J rearrangement and the lack of Pax5 expression in EBF-1 deficient mice place EBF-1 upstream of Pax5 (Lin, et al. 1995; Urbánek, Wang, Fetka, et al. 1994). This indicates that EBF-1 is a key factor for the specification and progression of the pro-B cell after entry of the cell into the B-lymphoid pathway but before final commitment of the cell due to Pax5 expression.

In addition to pro-B cells, EBF-1 is highly expressed in adipocytes (Lin, et al. 1995). EBF-1 appears to be involved in the regulation of the glucose transporter gene *Glut4* (Dowell and Cooke 2002). All three known O/E genes are expressed in mouse adipose tissue (Akerblad, Lind, Liberg, et al. 2002). Analysis of preadipocyte cell lines (3T3 L1) indicates the genes are expressed in undifferentiated cells and expression increases with differentiation (Akerblad 2002). Over expression enhances terminal adipocyte differentiation in preadipocyte cell lines and induces adipogenesis in mutipotential cells. The fact that O/E genes are expressed throughout adipocyte differentiation raises the possibility that they are key regulators of the pathway. However, the exact mechanism of how EBF-1 stimulates adipogenesis remains to be elucidated. The role of EBF-1 in adipocyte development in vivo is also unclear. Again, the fact that EBF-1−/− mice are smaller than their WT littermates may suggest a change in the balance between osteoblasts and adipocytes, reflected in bone.

3.1. Mouse Developmemt

EBF-1 deficient mice are maintained on the C57BL/6x129 backgrounds by heterozygous matings. At birth and until 10–14 days of age the EBF-1−/− pups were indistinguishable in size from their WT littermates (visual inspection). From that point on they lagged behind their control siblings in size and growth. We have observed mice for as long as 6 months and this runting persists. The EBF-1 mutant mice had normal tooth development and their eyes were normal suggesting functional osteoclasts were present. No other visible skeletal changes were observed.

3.2. Histology-Histomorphometry

In order to evaluate bone remodeling, the femora and the tibiae from 4 week-old EBF-1−/− mice were processed for histomorphometric analysis and evaluated by light microscopy. Age and sex matched littermates were used as controls. Histomorphometric measurements were performed on a fixed region just below the growth plate, corresponding to the primary spongiosa.

In control animals the primary spongiosa contained numerous spicules extending distally from the growth plate. These spicules formed the finger-like projections of the trabeculae and the resulting bone architecture was well organized and normal. Individual trabeculae were covered with thin seams of osteoid as would be anticipated for this period of high bone formation. Cortical bone was of normal thickness and still in the process of modeling/remodeling. The growth plate was orderly with normal appearing columns of developing chondrocytes. The bone marrow was unremarkable. The secondary center of ossification was well developed with a clear demarcation line at the growth plate. Bone marrow inside the secondary center was clearly viable and the entire structure was covered with a well-developed articular cartilage.

In contrast, the bones of homozygous mutant mice exhibited profound changes. The most striking feature was the adipocytes that filled the medullary canal. Interestingly, examination of the secondary center of ossification also showed the marrow space to be filled with fat cells. The growth plates of EBF-1−/− mice were similar to controls with no obvious abnormalities. Bones from EBF-1 mutant mice were physically smaller than controls. These data indicate that the loss of EBF-1 leads to marked adipogenesis in bones with marrow (Horowitz, Bothwell, Hesslein, Plugh and Schatz 2005).

The next obvious feature was the increased osteoblasts. The number of osteoblasts was strikingly increased compared to controls. Multiple layers of osteoblasts could be seen attached to bone surfaces. The inter-trabecular spaces at the growth plate were filled with osteoblasts while individual trabeculae were surrounded by cells. In fact, almost all of the formation parameters were increased significantly. These data not only indicate a marked increase in the number of osteoblasts but also suggest that they were functional. In addition, the bone formation rate was sharply increased.

Histomorphometric analysis also indicates that the number of osteoclasts in the mutant mice was reduced from that in controls. These data, taken as a whole, indicate markedly increased bone formation with increased osteoblasts and marrow adipogenesis.

In contrast to 4 week-old mice the bone volume was now significantly increased in 12 week-old EBF-1−/− mice as compared to controls. Osteoid volume and the number of osteoblasts remained high in the EBF-1 deficient mice. Importantly, fat persisted in filling the marrow space in a pattern similar to that seen in the 4 week-old mice. These data suggest that in EBF-1−/− mice an increase in osteoblast number starts early in life and persists as young adults. However, it takes time to develop a significant increase in bone mass. These data support our hypothesis that EBF-1 regulates osteoblast differentiation.

In addition to the increase in bone, the number of osteoclasts was markedly increased in 12-week-old mutant mice as compared to controls. This increase is even more striking in light of the data from the 4 week- old mice, in which the number of osteoclasts was decreased in the mutant mice. This suggests that although the number of osteoclasts was higher, and presumably, bone resorption was also increased, the amount of bone formation exceeded the resorption.

3.3. Osteoblast Function

Although EBF-1 expression is highly restricted to the B cell lineage, adipocytes, and olfactory neurons, no data is available on its expression in bone cells. To assess EBF-1 in bone cells, WT calvarial cells were grown to pre-confluent, confluent, and post-confluent density, total RNA collected, and EBF-1 expression determined by RT-PCR. Osteocalcin and Runx2/Cbfa1 expression were also determined as additional controls. As expected Runx2 was expressed by differentiating osteoblasts and mature cells expressed osteocalcin. Importantly,

EBF-1 was expressed at all stages of osteoblast differentiation. As reported previously, and confirmed here, B cells and fat also expressed EBF-1. In contrast, no EBF-1 mRNA could be detected in mature osteoclasts.

3.4. Splenic Characteristics

Dissection of the EBF-1−/− mice revealed spleens that were by inspection substantially smaller than controls. The low cell counts are in large part due to the lack of B cells, which constitute approximately 60% of normal spleen cells.

3.5. Osteoclast Development

The histomorphometric data indicate that the number of osteoclasts is decreased in 4 week-old EBF-1−/− bone, suggesting that in vitro production of osteoclasts should also be decreased. To test this possibility BM cells from mutant or control mice were cultured with M-CSF and RANKL for 7–10 days. The cultures were then fixed and stained for TRAP and the number of osteoclasts counted. The number of osteoclasts produced from 4 week-old EBF-1−/− BM cells was reduced by approximately half compared to control. In contrast, BM cells from 12-week-old mutant mice produced similar numbers of osteoclasts as controls. These data suggest that loss of EBF-1 causes an early decrease in osteoclast progenitors that recovers with age. These data also support the histomorphometric analysis. BM is comprised of >20% B lineage cells. EBF-1−/− BM is missing the majority of these cells. This loss of B cells serves to concentrate the remaining lineages within the BM including the osteoclast precursors. Therefore, the number of osteoclasts developing from mutant BM cells should be higher than in WT BM cells. However, this appears not to be the case in 4-week-old mice. The failure of EBF-1−/− BM to produce more osteoclasts than WT further supports the idea that loss of EBF-1 effects osteoclast development.

Culture of EBF-1 deficient spleen cells with RANKL and M-CSF induced similar numbers of osteoclasts as compared to WT cells. Culture of EBF-1 deficient spleen cells repeatedly failed to produce cell lines in a manner similar to Pax5.

3.6. Fat Phenotype

Because of the unusual increase in adipocytes seen in the marrow of EBF-1−/− mice, we examined other tissues from 4-week-old mutant and control mice for fat expression. Frozen sections were cut, and stained with Oil-red-O to identify fat. No difference in Oil-red-O staining was seen in spleen, heart, or skeletal muscle . However, a striking increase in Oil-red-O staining was observed in the livers of EBF-1−/− mice as compared to control. The liver data support the idea that EBF-1 may differentially regulate fat development (deposition), in a more global manner than we originally thought.

The mice have a “skin and bones” feel and appearance, suggesting they may be cachetic. To address this possibility, full thickness skin was recovered from the dorsal surface at mid-gut from 4-week-old EBF-1−/− and WT controls. The skin was fixed and stained with H&E. A comparison of WT and mutant skin revealed a number of differences. First, the dermis of the mutant mice is substantially thinner than WT. Second, the hair follicles in the mutant skin appear very small, strikingly smaller than WT. In addition, the hair shafts are thinner in the mutants.

Third, the subcutaneous fat is dramatically reduced in the EBF-1 deficient mice, accounting for the cachetic feel of the animals. These data support our idea that the EBF-1 −/− mice are lipodystrophic.

4. Pax5

Pax5 is a member of the multigene family that encodes the paired box (Pax) transcription factors. This highly conserved motif was originally identified in Drosophila (Bopp, Burri, Baumgartner, et al. 1986). At present, nine paired box containing genes (Pax1-Pax9) have been isolated in mammals (Walther, Guenet, Simon, et al. 1991; Burri, Tromvoulis, Bopp, et al. 1989). The transcription factors encoded by the Pax genes are 128 amino acids that recognize their target genes via the DNA binding function of the paired domain (Baumgartner, Bopp, Burri, et al. 1987). These genes are involved in the regulation of pattern formation and morphogenesis because they are expressed in distinct spatially and temporally restricted patterns during embryogenesis. At present three Pax genes have been studied in mice all of which exhibit developmental mutations. The Pax1 gene is mutated in different forms of *undulated*, which exhibit skeletal changes in the vertebra (Balling, Deutsch, and Gruss 1988). Mutations of Pax3 cause *Splotch*, which causes failure to close the neural tube, absence of limb musculature, and failure to develop certain neural crest derived tissue (Epstein, Vekemans, and Gros 1991). Pax6 mutations result in the Small eye mutation, which fail to develop eyes and nose (Hill, Favor, Hogan, et al. 1991). Whether any of these mutant mice express an altered bone phenotype is unknown. Human disorders have also been associated with mutations of Pax. Pax3 is mutated in Waardenburgs syndrome, which results in deafness, and Pax6 is changed in aniridia and in Peter's anomaly (Baldwin, Hoth, Amos, et al. 1992; Ton, Hirvonen, Miwa, et al. 1991). All of these mutations suggest the importance of Pax proteins in the specialization, proliferation, and migration of progenitor cells.

The Pax5 gene codes for the transcription factor B cell lineage specific activation factor (BSAP) (Adams, Dorfler, Aguzzi, et al. 1992). BSAP is the mammalian homologue of the sea urchin protein TSAP (tissue specific activation protein). During embryogenesis Pax5 is transiently expressed in the mesencephalon and spinal code in a pattern different from other Pax genes (Adams, Dorfler, Aguzzi, et al. 1992). Later in development, expression moves to the fetal liver where it correlates with the onset of B lymphopoiesis. Within the hematopoietic system, BSAP is expressed exclusively in the B lymphocyte lineage extending from pro-B cells to mature B cells but not in terminally differentiated plasma cells (Adams, Dorfler, Aguzzi, et al. 1992; Urbánek 1994). Testis is the only other tissue in the adult mouse that expresses BSAP.

4.1. Mouse Developmemt

Pax5 deficient mice are maintained on the C57BL/6 background by heterozygous matings. At birth and until 7–9 days of age the Pax5−/− pups were indistinguishable from their WT littermates. From that point on, they lagged behind their control siblings in size and growth. At 15 days the Pax5−/− mice were severely runted being approximately 1/3 the size of the controls. Few mice survive past 17 days of age. The cause of death is unknown. The Pax5 mutant mice had normal tooth development and their eyes were normal suggesting functional osteoclasts are present. No other visible skeletal changes were observed.

4.2. Histology-Histomorphometry

In order to evaluate bone remodeling, the femora and tibiae from 15-day-old Pax5−/− mice were processed for histomorphometric analysis and evaluated by light microscopy. Age and sex matched littermates (+/− heterozygous and +/+ homozygous C57BL/6 WT) were used as controls. Histomorphometric measurements were performed as described above. In control animals, the bone architecture was well organized and normal. Individual trabeculae were covered with osteoid as would be anticipated for this period of high bone formation. Cortical bone was of normal thickness and still in the process of modeling/remodeling. The bone marrow was unremarkable.

In contrast, the bones of homozygous mutant mice exhibited profound changes. The most striking feature was the dramatic osteopenia. Overall bone volume was reduced by approximately 66% compared to controls and osteoid volume was reduced by 55%. Trabecular thickness was reduced by 18%, the number of individual trabeculae was reduced by 51%, and the space between trabeculae, another indicator of bone resorption, was reduced by 61% in Pax5 deficient mice. Observed increases in bone resorption may be accounted for, at least in part, by the >100% increase in the number of osteoclasts in Pax5−/− bone (Horowitz, Xi, Pflugh, et al. 2004). Numerous osteoclasts were observed attached to bone spicules as compared to controls. These data not only indicate a marked increase in the number of osteoclasts but also suggest that they are functional. Histomorphometric analysis also indicates that the number of osteoblasts in mutant mice was reduced from that in controls although not significantly. The bone marrow from Pax5 deficient mice appears normal with no fibrosis. These data, taken as a whole, indicate markedly decreased trabecular bone due to the large increase in osteoclasts rather than a loss of osteoblasts.

In control animals, the growth plates were robust as would be expected during this early stage of life, with orderly columns of chondrocytes. In comparison the columns of chondrocytes appear compressed in the Pax5−/− mice. Examination of the secondary center of ossification shows little bone formation with numerous chondrocytes in mutant mice versus control. The Pax5 mutant mice also lack a seam of bone separating the growth plate from the secondary center. In addition, the articular cartilage in the Pax5−/− mice is thinned and the entire structure is flattened. These data suggest a delay in development, which could account, at least in part, for the runting.

4.3. Osteoblast Function

Although Pax5 expression is highly restricted to the B cell lineage no data is available on its expression in bone cells. To assess this possibility calvarial cells (>48 hrs. old) or cells isolated from adult long bones (LBC) of WT mice were grown to confluence, total RNA collected, and Pax5 expression determined by northern blot analysis. mRNA from a transformed pro-B cell line was used as a positive control. Neither calvarial nor LBC express Pax5.

To examine the possibility that the osteoblasts from the Pax5−/− mice may be altered the proliferative and alkaline phosphatase (ALP) response was measured over time. Both the proliferative response and the production of ALP by calvarial cells were similar in Pax5−/− and WT mice.

4.4. Splenic Characteristics

Dissection of the Pax5−/− mice revealed spleens that were by inspection substantially smaller than controls. Spleen weights were taken and show that Pax5 spleens were half the

weight of WT controls. The low cell counts are in part due to the lack of B cells, which constitute approximately 60% of normal spleen cells.

4.5. Osteoclast Development

One explanation for the histomorphometric data is that the Pax5−/− mice have increased numbers of osteoclast precursors. To test this possibility spleen cells from Pax5 deficient mice and controls were cultured with M-CSF and RANKL for 7–10 days. The cultures were then fixed and stained with TRAP and the number of osteoclasts counted. Pax5 spleen cells produced more than 3 fold the number of osteoclasts than WT spleen.

Culture of Pax5−/− spleen cells for 7-10 days resulted in the outgrowth of a population of adherent cells, which could be passaged in vitro multiple times (to date 1/week for > 7 weeks). Growth of the cells did not require added growth factors. To determine whether these cells could also be induced to form osteoclasts, cells were cultured in M-CSF and RANK-L, stained with TRAP and the number of osteoclasts counted. The Pax5−/− cell line consistently produced numerous osteoclast. Interestingly, it took only 3–4 days in culture to develop osteoclasts rather than the usual 7–10 days required for unselected spleen. These data show that an unusual population of cells, enriched in osteoclast precursors, spontaneously arises from Pax5−/− spleen. No equivalent cell line could be cultured from WT spleen.

4.6. Fat Phenotype

Pax5 deficient mice do not have a fat phenotype.

5. CONCLUSION

The data show that the EBF-1 and Pax5 deficient mice are similar in that they both lack essentially all B cells except those at the very earliest stages of development and are runted shortly after birth, although the runting in the Pax5 mutants is more severe. In contrast, numerous differences exist between the mutants. The Pax5−/− mice die by 18 days while the EBF-1−/− mice can survive for at least six months. The bone phenotype of the two mutants is completely different. The EBF-1−/− mice have increased bone mass with increased numbers of osteoblasts. Interestingly, the marrow is filled with adipocytes. The Pax5−/− mice are missing two thirds of their bone mass with a marked increase in the number of osteoclasts. A continuously growing cell line with cellular and molecular characteristics of osteoclast precursors can be grown from the spleen of Pax5−/− but not from EBF-1−/− mice. These differences are more striking because EBF-1 regulates Pax5 expression, with EBF-1−/− mice being deficient in Pax5. This suggests that the EBF-1 defect overrides the Pax5 induced changes. Although B cell deficiency is common to the two mutants little evidence has been developed showing that the loss of B cells contributes to the bone phenotype. In fact, mice made deficient in B cells by other mutations (Rag and mu deficiencies) do not exhibit bone phenotypes that at all resemble the EBF-1 or Pax5 bones. However, there is a strong correlation between loss of B cells and marked changes in the skeleton. Clearly, more work must be done to determine the true relationship of this association. What is clear is that these genes were originally identified and studied because of their importance to B cell differentiation. However, as more data is developed it becomes

apparent that EBF-1 and Pax5 are central to osteoblast and osteoclast differentiation. This observation is not dissimilar to the significance of PU.1 to macrophage, osteoclast, and B cell development. The logical extension of these ideas is that other transcription factors in the hierarchy of transcription factors required for B cell development will have important effects on bone cell growth and development. Ikarous would be a likely suspect (Georgopoulos, Bigby, Want, et al. 1994). It is clear that the skeletal and immune systems share numerous factors, receptors, cytokines, and signaling pathways. These molecules are important for the interaction and regulation of these two systems. The data support the idea that other, unrecognized proteins, that will also be important to these systems await to be discovered.

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CHAPTER 7
PTH REGULATES THE HEMATOPOIETIC STEM CELL NICHE IN BONE

Henry M. Kronenberg

1. INTRODUCTION

In late fetal life, hematopoiesis moves from the liver to the bone marrow in mammals, and continues to occur throughout postnatal life, with extramedullary hematopoiesis occurring only when disease blocks hematopoiesis in the marrow. Hematopoietic stem cells (HSCs) are uniquely the cells that can generate cells of all hematopoietic lineages. These cells do so by balancing an ability to increase their number by proliferation while staying pleuripotent (self-renewal) with their ability to differentiate into any of the hematopoietic lineages. Hematopoietic stem cells also happen to be able to circulate in the systemic circulation, a property likely to be important in their normal function. One of the reasons that we have learned so much about hematopoietic stem cells is that their properties underlie the ability of transplanted bone marrow to populate the marrow of patient recipients with failing bone marrow.

Clearly, the regulation of stem cell self-renewal and differentiation is crucial for normal function. A powerful idea that organizes a number of important properties of this regulation is that of the stem cell niche (Ohlstein, Kai, Decotto, et al. 2004; Scadden 2006). A niche is a particular place at which cells and matrix coalesce to regulate stem cells. This concept provides a source of the obvious need for paracrine regulation of stem cell properties. By providing a saturable number of sites for stem cell self-renewal, the niche also suggests a specific regulatory process: stem cells can enter and leave a niche and thus move toward or away from a unique site at which self renewal is possible. A recent series of advances has shown that hematopoietic stem cells can be found adjacent to both osteoblastic cells and endothelial cells in normal bone (Zhang, Niu, Ye, et al. 2003; Kiel, Yilmaz, Iwashita, et al. 2005). Here I will describe how activation of the PTH/PTHrP receptor found on cells of the osteoblast lineage can modify the niche for hematopoietic stem cells in bone in a way that allows such cells to accumulate in the marrow (Calvi, Adams, Weibrecht, et al. 2003). These findings have implications both for the normal regulation of hematopoiesis and for potential therapies in humans needing transplantation of human hematopoietic stem cells.

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2. MICE WITH CONSTITUTIVELY ACTIVE PTH/PTHrP RECEPTORS HAVE INCREASED NUMBERS OF HSCs

Patients with Jansen chondrodystrophy are hypercalcemic from the time of birth and subsequently develop short stature because of malfunction of the growth plates of all bones formed through endochondral bone formation. Schipani and Jüppner discovered that this striking combination of phenotypes is caused by point mutations in the receptor for both parathyroid hormone (PTH) and parathyroid hormone-related protein (PTHrP) (Schipani, Jensen, Parfitt, et al. 1996; Bastepe, Raas-Rothschild, Silver, et al. 2004). These mutations lead to constitutive activation of the receptor. As PTH receptors, these mutant receptors lead to hypercalcemia that mimics the hypercalcemia of hyperparathyroidism; as PTHrP receptors, these mutant receptors cause abnormal differentiation of growth plate chondrocytes that results in short stature. Calvi, Schipani, and co-workers used such mutant receptors as tools to understand the role of activation of the PTH/PTHrP receptor in cells of the osteoblast lineage (Calvi, Sims, Hunzelman, et al. 2001). They used a fragment of the collagen α1(I) gene that is expressed primarily in mature osteoblasts to drive expression of one version of the constitutively active PTH/PTHrP receptor in transgenic mice. The resultant mouse has a striking increase in trabecular bone mass at the ends of long bones (the metaphyseal region).

Calvi et al. asked whether the bones of mice expressing the constitutively active PTH/PTHrP receptor normally supported hematopoietic stem cells (Calvi, Adams, Weibrecht, et al. 2003). First, they showed that the population enriched for hematopoietic stem cells by cell sorting for characteristic markers ($Lin^{-}Sca\text{-}1^{+}kit^{+}$) is doubled in the transgenic mice compared to controls. Similarly, using a functional assay for cells behaving like HSCs *in vitro* (the long-term culture-initiating cell assay (LTC-IC)), the number of HSC-like cells was found to be increased. Most importantly, they then used the "gold standard" functional assessment of HSCs: competitive transplantation into irradiated hosts of marked wild type and marked cells from the transgenic mice. In this quantitative assay, the transgenic mouse had twice the normal number of HSCs capable of repopulating irradiated marrow. Thus, by all three assays, the osteoblasts of transgenic mice expressing a constitutively active PTH/PTHrP receptor only in cells of the osteoblast lineage support the accumulation of twice the normal number of HSCs. As expected from this formulation, when stromal cells isolated from the marrow of transgenic mice and from wild type mice were tested for their ability to support the production of LTC-IC using wild type marrow mononuclear cells, the transgenic stroma supported the accumulation of twice the normal number of LTC-IC.

A number of cytokines are known to support hematopoiesis. The transgenic stroma was found to contain increased amounts of immunoreactive interleukin 6 (IL-6), kit ligand or stem cell factor (SCF), and the chemokine stroma-derived factor 1 (SDF-1 or CXCL12). These and/or other diffusible cytokines cannot fully explain the properties of the stromal cells, however, because direct contact between the stromal and hematopoietic cells was required for the actions of the transgenic stroma to support increased numbers of LTC-IC. These cells also express increased amounts of immunoreactive Jagged-1, a membrane-bound ligand that activates the Notch signaling pathway. This is of particular interest because Notch signaling is known to stimulate self-renewal of HSCs. Consistent with the idea that the transgenic mice express more functional Jagged-1 than normal, Lin-Sca-1+Kit+ cells from the transgenic mice expressed higher than normal levels of the biologically active cleavage product of the Notch receptor. Importantly, inhibition of production of this cleavage product with an inhibitor of γ-secretase abolished the ability of the transgenic stroma to support LTC-IC more powerfully than wild type stroma. These experiments, taken together, support the idea that stromal cells in the transgenic mice support increased numbers of HSCs through the production of Jagged-1 and activation of the Notch pathway in HSCs. The roles of the production of diffusible cytokines might be

important as well, though the functional importance of these cytokines in mediating the effects of the transgene has not been evaluated.

3. PTH AND HSCs

To determine whether the actions of the constitutively active PTH/PTHrP receptor mimic the actions of parathyroid hormone on normal bone, PTH was administered to normal mice or added to cultures of normal bone stromal cells. In both the *in vivo* and the *in vitro* experiments, PTH increased the numbers of HSCs. Further, in the *in vitro* experiments, a γ-secretase inhibitor blocked the stimulation of HSC number just as it had in the experiments using transgenic stroma. These experiments establish that PTH (and perhaps PTHrP *in vivo*) stimulates the accumulation of HSCs through their activation of the PTH/PTHrP receptor.

This action of PTH, seen after administration of the low doses of PTH administered by once daily injection to increase bone mass, raises the possibility that PTH therapy might prove useful in the setting of bone marrow transplantation. To test this idea, a murine model was used in which syngeneic bone marrow mononuclear cells (BMMCs) are used to rescue the otherwise lethal effects of whole body irradiation. When purposely limited numbers of such BMMCs were used, most of the recipient mice died in the first two weeks after radiation. In contrast, when the same numbers of BMMCs were given to irradiated mice, but the mice were also treated with once daily injections of PTH, all of the mice survived and their marrows are repopulated with normal hematopoietic cells. This experiment thus raises the possibility that analogous therapy could prove useful in people receiving marrow transplants with limiting numbers of HSCs.

4. FUTURE DIRECTIONS

In independent work, Calvi's group has shown that the actions of PTH to increase HSCs is mediated by activation of the heterotrimeric Gs protein, the major G protein activated by the PTH/PTHrP receptor in many cell types (Weber, Forsythe, Christianson, et al. 2006). Further, using mice in which the α subunit of Gs is ablated specifically in osteoblasts (Wu, Kobayashi, Rodda, et al. 2006), Wu et al. showed that such mice have a dramatic defect in production of B cells in the marrow. These findings suggest that activation of Gs in cells of the osteoblast lineage may have different roles at different stages of hematopoiesis. An important focus of work in the future will be the characterization of the cells in the various niches that support HSCs and more differentiated progenitor hematopoietic cells. It seems likely that these cell types are not classical mature osteoblasts, cuboidal cells specialized to synthesize enormous amounts of type I collagen. Instead, these cells may be specialized cells of the osteoblastic lineage in the marrow stromal population (for B cell production) (Tokoyoda, Egawa, Sugiyama, et al. 2004) and on the surface of trabecular bone (for HSCs) (Zhang, Niu, Ye, et al. 2003; Arai, Hirao, Ohmura, et al. 2004; Suzuki, Ohneda, Minegishi, et al. 2006). How the differentiation and activity of these cells is regulated is an important question for the future.

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CHAPTER 8
REGULATION OF HEMATOPOIETIC STEM CELLS IN THE OSTEOBLASTIC NICHE

Fumio Arai and Toshio Suda

1. INTRODUCTION

Tissue stem cells are characterized by their abilities to self-renew and to produce numerous differentiated daughter cells. These two special properties enable stem cells to play a central role in maintaining tissues. Many adult tissue stem cells, including hematopoietic system, skin epidermis, gastrointestinal epithelium, brain, and lung were identified (Fuchs, Tumbar, and Guasch 2004; Moore and Lemischka 2006). The activity of tissue stem cells is crucial for supplying the mature cells in normal tissue turnover. It now clear that the stem cell niche regulates the stem cell-specific properties, including self-renewal activity, multi-potentiality, and relative quiescence (Suda, Arai, and Hirao 2005; Adams and Scadden 2006; Wilson and Trumpp 2006). Interaction of stem cells with stem cell niches is critical for maintaining the stem cell properties, including self-renewal capacity and the ability of differentiation into single or multiple lineages.

Hematopoietic stem cells (HSCs) are responsible for blood cell production throughout the lifetime of individual. BM HSCs are best-characterized stem cells. A small subset of HSCs is isolated by cell surface markers (Spangrude, Heimfeld, and Weissman 1988; Osawa, Hanada, Hamada, et al. 1996). These HSCs differentiate into myeloid cells, B cells, and T cells in the presence of various cytokines (Akashi, Traver, Miyamoto, and Weissman 2000). It has been reported that single purified HSC is able to reconstitute lethally irradiated mice (Osawa, Hanada, Hamada, et al.; Matsuzaki, Kinjo, Mulligan, et al. 2004). In contrast to the identification of HSCs, the localization of HSCs in situ and structure of HSC niche had not been solved. Recently, long-term bone marrow (BM) repopulating (LTR) HSCs have been found in BM trabecular bone surface, and it was clarified that an osteoblastic (OB) cell is a critical component for sustaining HSCs (Calvi, Adams, Weibrecht, et al. 2003; Zhang, Niu, Ye, et al. 2003). Long-term label retaining cell (LRC) study showed that 89 % of $CD45^{+}Lin^{-}$ LRCs attached to the endosteal surface (Zhang, Niu, Ye, et al. 2003). It suggests that quiescent/slow-dividing HSCs exclusively located in the osteoblastic niche. HSCs

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keep a balance between quiescence and cell division/proliferation in the osteoblastic niche (Arai, Hirao, Ohmura, et al. 2004). The specific properties of HSC are controlled dynamically by the signalings of receptor/ligand and cell adhesion molecules produced by osteoblastic niche cells (Suda, Arai, and Hirao 2005; Wilson and Trumpp 2006).

We described here the characterization of HSC and their niche, and the environmental regulation of HSCs in the niche.

2. STEM CELL NICHE AND NICHE CELLS IN ADULT BM

Stem cell niche is the specialized microenvironments regulate stem cell behavior. The stem cell niche concept was first proposed for the human hematopoietic system in the 1970's (Schofield 1978). Niche is sometimes used to describe the stem cells location, the definition of the niche is that niche is composed of the cellular components surrounding stem cells and signaling molecules provided by supporting cells (Lin 2002).

Recently, it has been reported that the LTR VHSCs exist frequently trabecular bone surface in BM (Calvi, Adams, Weibrecht, et al. 2003; Zhang, Niu, Ye, et al. 2003; Arai, Hirao, Ohmura, et al. 2004). And it was clarified that bone-lining OBs are critical regulatory component of adult hematopoiesis. Studies using genetic strategies showed that the number of LTR-HSCs was dependent on increase or decrease the OB population in BM (Calvi, Adams, Weibrecht, et al. 2003; Zhang, Niu, Ye, et al. 2003; Visnjic, Kalajzic, Rowe, et al. 2004). Increasing the number of OBs causes parallel increases in the HSC population, particularly LTR-HSCs, without concomitant increases in other primitive progenitor cells. Such a specific increase in only the LTR-HSC population suggests that a specific niche is functionally enhanced (Calvi, Adams, Weibrecht, et al. 2003; Zhang, Niu, Ye, et al. 2003). On the contrary, the depletion of OBs using a type I collagen promoter-herpes simplex virus thymidine kinase transgenic mice with ganciclovir treatment led to reduction of BM cellularity (Visnjic, Kalajzic, Rowe, et al. 2004). OBs constitute part of the stromal cell support in BM. Taichman and Emerson also reported that OBs are a part of the hematopoietic microenvironment after BM cavity formation (Taichman and Emerson 1998). Nilsson et al. reported that HSCs were significantly enriched within the endosteal region after BM transplantation (Nilsson, Johnston, and Coverdale 2001). We previously reported that OBs express angiopoietin-1 (Ang-1), and maintained HSCs *in vitro* (Arai, Ohneda, Miyamoto, et al. 2002).

3. QUIESCENT HSCs IN THE ADULT BM NICHE

The key features of stem cells in a niche are that they are quiescent and adhere to surrounding niche cells (Lin, 2002; Tumbar, Guasch, Greco, et al. 2004; Blanpain, Lowry, Geoghegan, et al. 2004; Yamashita, Jones, and Fuller 2003). The quiescence of stem cells is of critical biologic importance in protecting the stem cell compartment (Cheng, Rodrigues, Shen, et al. 2000). It has been reported that HSCs are relatively quiescent when compared to transiently amplifying progenitor cells (Allsopp, Morin, DePinho, et al. 2003). In this study, we analyzed side-population (SP) cells in c-Kit$^+$Sca-1$^+$Lineage$^-$ (KSL) HSCs under 5-fluorouracil (5-FU) induced myelosuppressive condition. SP is a cell fraction weakly or non-labeled with the DNA dye, Hoechst 33342, and is able to enrich the HSCs (Goodell, Brose, Paradis, et al. 1996; Goodell, Rosenzweig, Kim, et al. 1997). Moreover, SP cells have been found in several tissue and

species, suggesting that SP defines a general property of tissue stem cells (Zhou, Schuetz, Bunting, et al. 2001). We found that the SP cells, but not non-SP cells in KSL are resistant to BM suppression induced by 5-FU (Arai, Hirao, Ohmura, et al. 2004). Since 5-FU induces apoptosis of actively cycling cell, our finding indicated that KSL-SP are quiescent and have anti-apoptotic properties. In addition, we investigated the cell cycle status of SP cells by pyronin Y (PY) staining. PY forms the fluorescent complexes with double-stranded RNA and DNA. In the presence of a DNA-binding dye such as a Hoechst 33342, PY stains double-stranded RNA specifically and quantitatively (Kapuscinski and Darzynkiewicz 1987). We confirmed that most KSL-SP cells (92.4 ± 6.7 %) were $PY^{low/-}$, suggesting that KSL-SP cells are in G0 phase of cell cycle. It was previously reported that $PY^{low/-}$ and PY^{+} cells were in G0 and G1 phases of cell cycle, respectively (Hüttmann, Liu, Boyd, et al. 2001). Therefore we believe that SP is the most suitable marker for detection of quiescent HSCs.

To identify quiescent HSCs in situ, we analyzed specific markers for KSL-SP cells. We found that SP cells were specifically enriched in Tie2 receptor tyrosine kinase-expressing KSL cells, and $Tie2^{+}$ KSL cells survived after 5-FU treatment, as did SP cells (Arai, Hirao, Ohmura, et al. 2004). Therefore we analyzed the expression of Tie2 in 5-FU treated BM, and we found that $Tie2^{+}$ HSCs adhered to OBs at the surface of the trabecular bone. The findings were in agreement with those previously reported (Calvi, Adams, Weibrecht, et al. 2003; Zhang, Niu, Ye, et al. 2003). Ang-1, a ligand for the Tie2 receptor is mainly produced by OBs. This finding suggests that Tie2 and Ang-1 are expressed complementarily in the niche. Taken together, our observations support the idea that the niche is a microenvironment reserved for quiescent HSCs. HSCs thus may transit between niche and non-niche sites and/or between quiescence and active cell cycling in vivo. When this balance is disrupted, as occurs with $p21^{WAF1/Cip1}$ deficiency, HSCs cannot remain in G0 and long-term repopulating ability is lost (Cheng, Rodrigues, Shen, et al. 2000). This finding indicates that the niche is essential for maintenance of a long-term hematopoietic system.

4. ROLE OF Tie2/Ang-1 SIGNALING IN ADULT HEMATOPOIESIS

4.1. Tie2/Ang-1 Signaling

Tie2 is member of Tie receptor tyrosine kinase family (Tie1 and Tie2) and is a expressed on endothelial cells (ECs) and HSCs (Dumont, Yamaguchi, Conlon, et al. 1992; Partanen, Armstrong, Makela, et al. 1992; Iwama, Hamaguchi, Hashiyama, et al. 1993). Tie1 and Tie2 have unique structural properties: two immunoglobulin-like domains, three epidermal growth factor (EGF)-like domains, and three fibronectin type III-like repeats in the extracellular region and a split catalytic domain in the cytoplasmic region. The functions of Tie2 has mainly been analyzed in ECs. Tie2-deficient mice show abnormal vascular network formation (Dumont, Gradwohl, Fong, et al. 1994; Sato, Tozawa, Deutsch, et al. 1995). Moreover, an activating mutation in Tie2 causes vascular dysmorphogenesis in humans (Vikkula, Boon, Carraway, et al. 1996).

Ang-1, has been identified by a secretion-trap expression cloning strategy (Davis, Aldrich, Jones, et al. 1996). The nucleotide sequence of Ang-1 cDNA contains an open reading frame encoding 498 amino acids including a coiled-coil domain and a fibrinogen-like domain. Although Ang-1 does not affect EC growth in culture, mice lacking Ang-1 display angiogenic deficits similar to Tie2 deficient mice (Suri, Jones, Patan, et al. 1996).

It was reported that Ang-1 promotes adhesion of Tie2 HSCs to fibronectin (FN) and collagen (COL) (Takakura, Huang, Naruse, et al. 1998; Sato, Iwama, Takakura, et al. 1998) and also promotes the interaction of ECs with surrounding mesenchymal cells and the extracellular matrix (Davis, Aldrich, Jones, et al. 1996; Suri, Jones, Patan, et al. 1996). We found that Tie2/Ang-1 signaling activates β1-integrin and N-cadherin in Tie2 HSCs, and promotes HSC interactions with extracellular matrix and cellular components of the niche (Arai, Hirao, Ohmura, et al. 2004). Zhang et al. found that spindle-shaped N-cadherin-positive OBs (SNO cells) are niche cells, and N-cadherin is asymmetrically localized between HSCs and OBs in the adult BM niche (Zhang, Niu, Ye, et al. 2003). These data suggest that N-cadherin is a key downstream target of Tie2/Ang-1 signaling in HSCs. It was reported that c-Myc deficient HSCs show up-regulation of cell adhesion molecules, such as N-cadherin and integrins, and these cells cannot detach from the niche. In contrast, forced expression of c-Myc in HSCs represses N-cadherin and integrins leading to loss of self-renewal activity (Wilson, Murphy, Oskarsson, et al. 2004; Murphy, Wilson, and Trumpp, et al. 2005). It suggests that release of HSCs from the niche requires c-Myc activity. In addition, these observations raise the possibility that c-Myc is a negative mediator downstream of Tie2/Ang-1. From these findings, we hypothesized that the localization of quiescent HSCs on the bone surface is regulated by stem cell specific adhesion molecules such as N-cadherin. Once the HSCs localize to SNO cells, Ang-1 produced by SNO cells may activate Tie2 on HSCs and promote tight adhesion of HSCs in the niche.

HSCs lose their self-renewal activity after repeated cell divisions in culture in vitro (Ema, Takano, Sudo, et al. 2000). We found that Tie2/Ang-1 signaling inhibits HSC division in culture, resulting in the maintenance of LTR-activity of HSCs after in vitro culture. In addition, exogenous Ang-1 dramatically increased the proportion of SP cells in the KSL population indicating that increment of quiescent HSCs (Arai, Hirao, Ohmura, et al. 2004). Tie2 signal induced quiescence is potentially caused by positively regulating p21. p21 is an essential molecule for maintenance of HSC quiescence (Cheng, Rodrigues, Shen, et al. 2000). It was reported p21 transcription is negatively regulated by c-Myc (Wu, Cetinkaya, Munoz-Alonso, et al. 2003).

Genetic evidence for the importance of Tie2 signaling in HSC-niche interaction has been clarified by the study of chimeric mice composed of both normal embryonic cells and Tie receptor Tie1/Tie2-deficient cells. Although Tie1 and Tie2 receptors are not required for the fetal hematopoiesis, including the emergence of definitive HSCs, or for relocation to and differentiation in the fetal liver, HSCs lacking both Tie1 and Tie2 fail to be maintained in the adult BM microenvironment (Puri and Bernstein 2003). Tie1-deficient cells, which express normal levels of Tie2, contribute to hematopoiesis (Partanen, Puri, Schwartz, et al. 1996; Rodewald and Sato 1996). This finding indicated that Tie2 is required for postnatal BM hematopoiesis but not for embryonic hematopoiesis. In addition, analysis of chimeric mice that included Tie receptor-deficient donors and $Rag2^{-/-}$ hosts, which do not produce mature lymphocytes, showed that Tie2/Tie1-deficient cells contribute to lymphopoiesis in the absence of competing host cells (Puri and Bernstein 2003). We also demonstrated that kinase dead-mutant Tie2 (KD-Tie2)-expressing HSCs could not maintain quiescence and did not express the SP phenotype (Arai, Hirao, Ohmura, et al. 2004). These findings strongly suggest that Tie2 is critical for the maintenance and survival of HSCs in adult BM and that Tie2-deficient or KD-Tie2 cells are unable to occupy the adult BM niche when competing with wild-type cells.

Further studies may reveal other molecules or signaling pathways required for cell adhesion and cell cycle regulation by niche factors. Understanding of factors underlying cell adhesion and cell cycle regulation in stem cells should lead to development of new strategies for regenerative medicine.

4.2. Regulation of HSCs by Reactive Oxygen Species

Recently, we found that ataxia telangiectasia mutated (ATM) regulates the LTR-activity of HSCs but not their proliferation or differentiation into progenitors (Ito, Hirao, Arai, et al. 2004). ATM maintains genomic stability by activating a key cell cycle checkpoint in response to DNA damage, telomeric instability or oxidative stress. $Atm^{-/-}$ mice over the age of 24 weeks show progressive BM failure due to a defect in HSC function associated with elevated reactive oxygen species (ROS), but they do not show telomere dysfunction. Elevation of ROS activated p38MAPK in HSCs and phosphorylation of p38MAPK induced upregulation of the cyclin-dependent kinase (CDK) inhibitor $p16^{Ink4a}$ and the retinoblastoma (Rb) gene in $Atm^{-/-}$ HSCs (Ito, Hirao, Arai, et al. 2004, 2006). In addition, the proportion of SP cells and $PY^{low/-}$ cells were significantly lower than that seen in wild-type mice, suggesting that $Atm^{-/-}$ HSCs were not in quiescent state. Treatment with anti-oxidative reagents or with MAPK inhibitor restores the reconstitutive capacity and quiescence of $Atm^{-/-}$ HSCs (Ito, Hirao, Arai, et al. 2006).

These data suggest that regulation of oxidative stress is critical for the maintenance of stem quiescence. Although it is still unclear that the effects of oxidative stress in the maintenance of HSC-niche interaction, elevation of ROS in HSCs may affect the interaction of HSCs with the niche and lead to the loss of quiescence.

Now we speculate that niches or niche cells for quiescent stem cells are located in hypoxic regions of BM, such as the trabecular bone surface, where they not only keep stem cells quiescent through cell-adhesion but also protect them from ROS.

5. CONCLUSION

Regulation of cell cycle quiescence and cell adhesion of HSCs by the niche is critical for the maintenance of HSCs. Signaling molecules, extracellular matrix, and cell adhesion molecules produced by niche cell regulate quiescence, self-renewal, and cell fate decision of the HSCs. Our data clearly demonstrate that quiescent Tie2 HSCs specifically localized in the BM niche and adhere to the bone-lining OBs. Tie2 HSCs in adult BM meet the following criteria for being a tissue stem cell: quiescence, anti-apoptosis, and an SP phenotype. Our presentation shows a novel mechanism for regulation of the quality of stem cell that is regulated by the microenvironment. In this model, Ang-1 produced by OBs activates Tie2 on the HSCs and promote tight adhesion of HSCs to the niche, resulting in quiescence and enhanced survival of HSCs.

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CHAPTER 9
THE CHEMOKINE CXCL12 AND REGULATION OF HSC AND B LYMPHOCYTE DEVELOPMENT IN THE BONE MARROW NICHE

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1. ABSTRACT

Chemokines are a family of small structurally related molecules that were recognized originally for their ability to regulate cell trafficking in inflammation. We have found that a chemokine, CXC chemokine ligand 12/stromal cell-derived factor/pre-B-cell growth stimulating factor (CXCL12/SDF-1/PBSF) and its physiologic receptor CXCR4 are essential for hematopoiesis including B lymphocyte development and colonization of bone marrow by hematopoietic cells including hematopoietic stem cells (HSCs) during ontogeny as well as cardiovascular formation. Recently, we have shown that a small population of reticular stromal cells, which has high levels of CXCL12 expression, termed CXCL12-abundant reticular (CAR) cells have several long processes and are scattered throughout adult bone marrow. In addition, most of the earliest B cell precursors, pre-pro-B cells and end-stage B cells, plasma cells, which require CXCL12, as well as primitive hematopoietic progenitors were attached to the CAR cells. These results suggest that the CAR cells function as cellular niches for B-cell development and that CXCL12 plays a role in maintaining the blood cells in the niches. It has been hypothesized that osteoprogenitors reside in the stromal tissues of bone marrow and play an important role in hematopoiesis. The nature and functions of CAR cells are important issues for the future.

2. INTRODUCTION

In mammals, haematopoiesis takes place within a complex microenvironment of bone marrow, which occupies the medullary cavities of bones. In the bone marrow, diverse lineages of haematopoietic precursors arise from hematopoietic stem cells (HSCs) and develop in the

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densely cellular extravascular spaces between a network of medullary vascular sinuses. In addition, it has been assumed that the bone marrow contains adherent cells, known as stromal cells, which create special microenvironment termed niches, that maintain blood cell viability and supply the requisite factors for their development. Thus, spatial organization of blood cells and their interaction with the stromal cell niches within the bone marrow are important for in the regulation of haematopoiesis.

It has been previously reported that, during development, haematopoietic cells migrate from the subendosteal region (the inner bone surface) towards the central region of the bone marrow cavity (Lord, Testa and Hendry 1975). Consistent with this, some groups have reported that primitive haematopoietic progenitors are localized in close contact with the endosteum (Calvi, Adams, Weibrecht, et al. 2003; Zhang, Niu, Ye, et al. 2003). In vivo studies have suggested that osteogenic cells are essential regulators of the development of all blood cells in bone marrow and that they act as niches for HSCs (Calvi, Adams, Weibrecht, et al. 2003; Zhang, Niu, Ye, et al. 2003; Visnjic, Kalajzic, Rowe, et al. 2004). By contrast, others have shown that primitive haematopoietic progenitors including HSCs are uniformly scattered throughout the bone marrow (Hirose, Kouro, Igarashi, et al. 2002; Tokoyoda, Egawa, Sugiyama, et al. 2004; Kiel, Yilmaz, Iwashita, et al. 2005). So, the identification of the specific stromal cell niches is of importance in understanding blood cell behavior within bone marrow. However, the lack of distinctive characteristics of stromal cells in bone marrow has made it difficult to identify the niches for HSCs and precursors of various haematopoietic lineages. To address this issue, we focused our analysis on the roles of a cytokine, CXC chemokine ligand (CXCL)12 and the specialized stromal cells which expressed CXCL12 at high levels and resided in bone marrow (Tokoyoda, Egawa, Sugiyama, et al. 2004; Nagasawa 2006).

3. CXCL12 AND ITS PHYSIOLOGIC RECEPTOR CXCR4

CXCL12 is a cytokine which belongs to a chemokine superfamily. Chemokines are a large family of structurally related chemoattractive cytokines, which act via a seven transmembrane-spanning G protein-coupled receptors (GPCR) and were recognized originally for their ability to regulate cell trafficking in inflammation (Baggiolini, Dewald and Moser 1997). CXCL12 also known as stromal cell-derived factor(SDF)-1/pre-B-cell-growth-stimulating factor (PBSF) is the first chemokine that has been shown to be critical for various developmental processes including hematopoiesis (Nagasawa 2006; Nagasawa, Hirota, Tachibana, et al. 1996). CXCL12 was isolated from stromal cell lines (Tashiro, Tada, Heilker, et al. 1993; Nagasawa, Kikutani, and Kishimoto 1994) and first characterized as a growth stimulating factor for a stromal cell dependent B cell precursor clone (Nagasawa, Kikutani, and Kishimoto, et al. 1994). The primary physiologic receptor for CXCL12 is CXCR4 (Nagasawa 2006; Nagasawa, Hirota, Tachibana, et al. 1996; Tachibana, Hirota, Iizasa, et al. 1998; Zou, Kottmann, Kuroda, et al. 1998), which also functions as an entry receptor for strains of HIV-1 (Nagasawa 2006). The studies using mutant mice with targeted gene disruption have revealed that CXCL12 and CXCR4 are essential for embryonic viability (Nagasawa, Hirota, Tachibana, et al. 1996; Tachibana, Hirota, Iizasa, et al. 1998; Zou, Kottmann, Kuroda, et al. 1998) B cell development (Nagasawa, Hitrota, Tachibana, et al. 1996; Tachibana, Hirota, Iizasa, et al. 1998; Egawa, Kawabata, Kawamoto, et al. 2001), colonization of bone marrow by HSCs, primitive hematopoietic progenitors and myeloid lineage cells (Nagasawa, Hirota, Tachibana, et al. 1996; Tachibana, Hirota, Iizasa, et al. 1998; Zou, Kottmann, Kuroda, et al. 1998; Ara, Tokoyoda, Sugiyama, et al. 2003), colonization of gonads by primordial germ cells (PGCs) into gonads during ontogeny (Raz 2003), blood vessel

formation in gastrointestinal tract (Tachibana, Hirota, Iizasa, et al. 1998)[13], cardiac ventricular septum formation (Nagasawa, Hirota, Tachibana, et al. 1996; Tachibana, Hirota, Iizasa, et al. 1998; Zou, Kottmann, Kuroda, et al. 1998) and development of the cerebellum (Zou, Kottmann, Kuroda, et al. 1998; Tran, and Miller 2003), hindbrain (Lieberam, Agalliu, Nagasawa, et al. 2005), spinal cord (Lieberam, Agalliu, Nagasawa, et al. 2005), hippocampus (Tran, and Miller 2003) and neocortex (Tran, and Miller 2003). Furthermore it has been reported that CXCR4 is involved in retaining hematopoietic precursors in hematopoietic organs (Ma, Jones, and Springer 1999). Consistent with these, the studies using transfilter migration and transplantation assay have revealed that various kinds of cells including hematopoietic precursors migrate in response to CXCL12 in vitro and in vivo (Nagasawa 2006; Bleul, Fuhlbrigge, Casasnovas, et al. 1996; Peled, Petit, Kollet, et al. 1999).

4. THE ROLES OF CXCL12-CXCR4 SIGNALING IN B CELL DEVELOPMENT

In B cell development, CXCL12-deficient embryos contained severely reduced numbers of $Lin^{-}CD19^{-}c\text{-}kit^{+}IL\text{-}7R^{+}AA4.1^{+}$ earliest B cell precursors in fetal liver and the numbers of B cell precursors including the earliest pre-pro-B cells are severely decreased in adult bone marrow in chimeric mice reconstituted by $CXCR4^{-/-}$ fetal liver cells compared with control chimeras, indicating that CXCL12-CXCR4 signaling is essential for the earliest stages in fetal and adult B cell development (Egawa, Kawabata, Kawamoto, et al. 2001) (Figure 1). B cell development is believed to proceed from pre-pro-B cells to pro-B to pre-B cells (Hardy, Carmack, Shinton, et al. 1991).

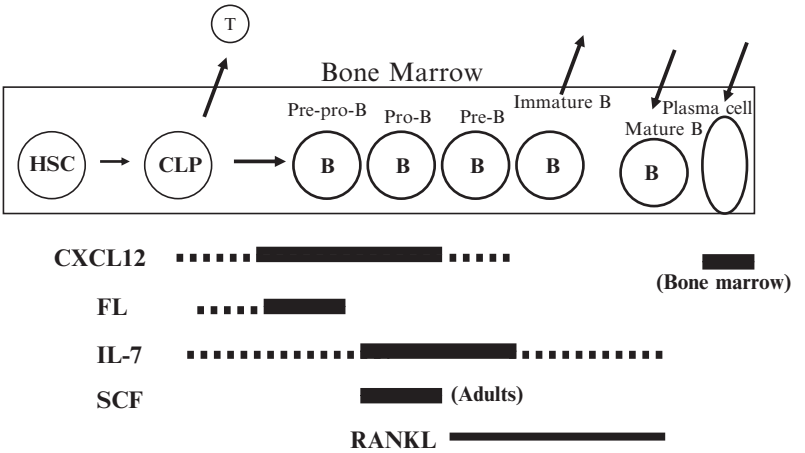

Figure 1. A pathway for early B-cell differentiation and cytokine requirement in bone marrow B cells. The differentiation pathway is thought to proceed from pre-pro-B to pro-B to pre-B cells (Hardy, Carmack, Shinton, et al. 1991). Immature B cells are generated from pre-B cells, and exit the bone marrow and reach the spleen, where they mature into peripheral mature B cells. Plasma cells develop following antigen activation of mature B cells. CXCL12 is essential for generation of pre-pro-B and pro-B cells (Egawa, Kawabata, Kawamoto, et al. 2001), and homing of plasma cells to the bone marrow (Tokoyoda, Egawa, Sugiyama, et al. 2004; Hargreaves, Hyman, Lu, et al. 2001). FLT3L is essential for generation of pre-pro-B cells (Nagasawa 2006). IL-7 is essential for generation of pro-B and pre-B cells but not pre-pro-B cells (Nagasawa 2006). The recent study has shown that IL-7 is required for B-cell differentiation potential of pre-pro-B cells (Nagasawa 2006). SCF is essential for pro-B cells in adults (Nagasawa 2006). RANKL is involved in the generation of pre-B cells and immature B cells (Nagasawa 2006).

It has been reported that the numbers of pre-B cells are normal in bone marrow but significantly increased in the peripheral blood in conditional CXCR4 deficient mice in which CXCR4 was specifically deleted in late stages of B lineage cells ($CXCR4^{flox/-}$/CD19-Cre mice), compared with control animals, suggesting that CXCR4 is involved in retaining pre-B cells in bone marrow (Nie, Waite, Brewer, et al. 2004). B lymphocytes positive for surface immunoglobulin which develop from pre-B cells in bone marrow egress into peripheral blood to reach the spleen and mature to peripheral mature B cells. Plasma cells develop following antigen activation of mature B cells and the majority of long-lived plasma cells which are important for immunological memory again migrate to bone marrow. The studies using $CXCR4^{flox/-}$/CD19-Cre mice or chimeric mice reconstituted with $CXCR4^{-/-}$ fetal liver cells have shown that CXCL12-CXCR4 signaling plays a critical role in homing of plasma cells specifically into bone marrow (Tokoyoda, Egawa, Sugiyama, et al. 2004; Hargreaves, Hyman, Lu, et al. 2001).

5. CXCL12-ABUNDANT RETICULAR CELLS

The result that a member of chemoattractive cytokine family CXCL12 is required for the earliest B cell precursors raises the possibility that CXCL12 attracts and/or tethers precursors immediately after commitment to the B lineage in an appropriate microenvironment comprising cells expressing CXCL12. Thus we analyzed the localization of cells expressing CXCL12 in bone marrow using the mice with the GFP reporter gene knocked into the CXCL12 locus (CXCL12/GFP knockin mice) (Tokoyoda, Egawa, Sugiyama, et al. 2004). Strong CXCL12/GFP expression was seen in a small population of stromal cells and these cells had several processes like the fibroblast-like cells morphologically termed reticular cells (Tokoyoda, Egawa, Sugiyama, et al. 2004; Jacobsen, Kravitz, Kincade, et al. 1996). The cells expressing CXCL12 at high levels, termed reticular CXCL12-abundant, (CAR) cells occurred singly and were uniformly scattered throughout the bone marrow (Tokoyoda, Egawa, Sugiyama, et al. 2004). Within bone marrow, reticular cells as well as sinusoid endothelial cells have been shown to express vascular cell adhesion molecule (VCAM)-1 within bone marrow (Hargreaves, Hyman, Lu, et al. 2001). Immunohistochemical analysis has revealed that almost all of CAR cells expressed VCAM-1 and constitute approximately 20% of $VCAM\text{-}1^+$ cells, supporting the idea that CAR cells is a subset of bone marrow reticular cells. In addition, almost all of CAR cells do not express PECAM-1 or the markers for osteoblasts including osteopontin and osteocalcin, suggesting that CAR cells are different from endothelial cells and osteoblasts. Consistent with these, CAR cells are located some distance from the bone surface.

6. ASSOCIATION OF CAR CELLS WITH B LYMPHOCYTES AND PRIMITIVE HEMATOPOIETIC CELLS

Next, we examined the association of CAR cells with B lineage cells (Tokoyoda, Egawa, Sugiyama, et al. 2004). Immunohistochemical analysis revealed that most $B220^+flk2^+$ Fraction A cells, which include pre-pro-B cells (Hargreaves, Hyman, Lu, et al. 2001) were in contact with CAR cells (Tokoyoda, Egawa, Sugiyama, et al. 2004). In contrast, many $B220^+c\text{-}kit^+$ pro-B, $B220^+IL\text{-}7R^+$ pre-B and $B220^+IgM^+$ immature B cells were located at a distance from CAR cells (Tokoyoda, Egawa, Sugiyama, et al. 2004). Immunohistological analysis using antibodies against IgG and the plasma cell marker, Syndecan-1 revealed that most of $IgG^+Syndecan\text{-}1^+$ plasma cells were in contact with CAR cells (Tokoyoda, Egawa, Sugiyama, et al. 2004).

Since B cell precursors are generated directly or indirectly from multipotent hematopoietic progenitors including HSCs or common lymphoid progenitors (CLPs), we tried to determine the association of multipotent hematopoietic progenitors with CAR cells (Tokoyoda, Egawa, Sugiyama, et al. 2004). It remains difficult to visualize HSCs or CLPs. However, since the c-kit^{+}Sca-1 $^{+}$ cells are thought to be highly enriched in variety of multipotent hematopoietic progenitors, immunohistochemical staining analysis using antibodies against c-kit and Sca-1 was performed to visualize multipotent hematopoietic progenitors (Tokoyoda, Egawa, Sugiyama, et al. 2004). C-kit^{+}Sca-1^{+} cells were observed throughout bone marrow and most c-kit^{+}Sca-1^{+} cells were in contact with the course of the processes of CAR cells (Tokoyoda, Egawa, Sugiyama, et al. 2004).

7. CLOSING REMARKS

Since pre-pro-B and plasma cells which require CXCL12 are in contact with some CAR cells within bone marrow, CAR cells would constitute a specific niche for precursors at the earliest and end stages of B lymphopoiesis (Tokoyoda, Egawa, Sugiyama, et al. 2004; Nagasawa 2006) (Figure 2). In addition, CXCL12 is a candidate for the environmental factors which play a role in maintaining the precursors in their niche.

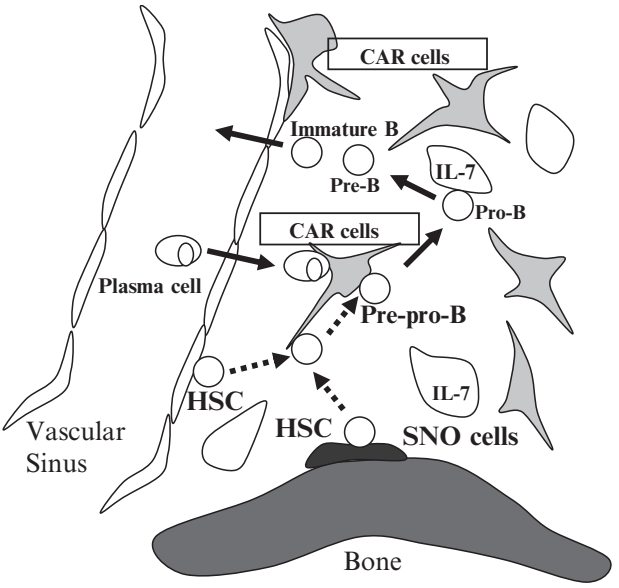

Figure 2. CAR cells are candidates for cellular niches for B cell development and primitive hematopoietic progenitors. CAR cells occurred singly and are uniformly scattered throughout the bone marrow extravascular spaces (Tokoyoda, Egawa, Sugiyama, et al. 2004). In this model, progeny of HSCs located near the bone surface (Calvi, Adams, Weibrecht, et al. 2003; Zhang, Niu, Ye, et al. 2003; Arai, Hirao, Ohmura, et al. 2004) would move toward the CAR cells. Primitive hematopoietic progenitors and the earliest pre-pro-B cells are maintained in contact with CAR cells (Tokoyoda, Egawa, Sugiyama, et al. 2004). End-stage B cell, plasma cells again seed CAR cells (Tokoyoda, Egawa, Sugiyama, et al. 2004). HSC, hematopoietic stem cells.

Committed B cell precursors are thought to generate from multipotent hematopoietic progenitors including CLPs and HSCs. Considering that CAR cells adjacent to endothelial cells might act as a hematopoietic stem cell niche in fetal bone marrow (Ara, Tokoyoda, Sugiyama, et al. 2003) and that many HSCs are associated with endothelium in bone marrow (Kiel, Yilmaz, Iwashita, et al. 2005), there is the possibility that HSCs remain adjacent to CAR cells in adult bone marrow. The results that most c-kit$^+$Sca-1$^+$ cells adhere to the CAR cells (Tokoyoda, Egawa, Sugiyama, et al. 2004) support the possibility. In this case, commitment to B lymphoid lineage occurs adjacent to CAR cells. On the other hand, recent studies suggest that HSCs are located near the osteoblastic cells termed SNO cells lining the bone surface (Calvi, Adams, Weibrecht, et al. 2003; Zhang, Niu, Ye, et al. 2003; Arai, Hirao, Ohmura, et al. 2004). In this case, intermediates between HSCs and B cell precursors would move from the bone surface toward the CAR cells, which are located some distance from the bone surface (Figure 2).

What is the lineage nature of CAR cells? Reticular cells in bone marrow have been thought to be fibroblastic in nature. However, there is the possibility that CAR cells are progenitors of the osteogenic lineage since it has been reported previously that osteogenic lineage cells with long processes are present in the intertrabecular space of bone marrow (Rouleau, Mitchell, and Goltzman 1990). The phenotypic characterization of CAR cells and their functions in spatiotemporal regulation of hematopoiesis and bone formation are important issues for the future.

8. ACKNOWLEDGMENTS

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CHAPTER 10
OSTEOCLAST PRECURSOR CELLS

Joseph Lorenzo, M.D.

1. ORIGIN OF THE OSTEOCLAST

The hematopoietic origin of the osteoclast is now clear. Walker (Walker 1975b, Walker 1975a, Walker 1975c) first demonstrated that the precursor cells of osteoclasts were hematopoietic. These studies showed that the transplant of spleen cells from osteopetrotic mice, which have dysfunctional osteoclasts, into irradiated normal animals caused the normal animals to become osteopetrotic within four weeks. Conversely, it was shown that transplant of normal spleen cells into irradiated osteopetrotic mice caused the osteopetrotic mice to develop normal bone remodeling within the same time period. More recently, bone marrow transplants into humans with osteopetrosis have, in selected cases, led to reversal of the condition (Coccia, Krivit, Cervenka, et al. 1980).

Testa et al. first illustrated that feline bone marrow cultures could be induced to form multinucleated osteoclast-like cells (OCL) (Testa, Allen, Lajtha, et al. 1981, Allen, Testa, Suda, et al. 1981). Subsequently, Ibbotson et al characterized this system and demonstrated that stimulators of bone resorption enhanced the rate of formation of OCL (Ibbotson, Roodman, McManus, et al. 1984). Culture systems that use human or mouse marrow have been extensively studied and these demonstrate that the OCL that form have many characteristics of true osteoclasts. These include the abundant production of tartrate resistant acid phosphatase (TRAP) and calcitonin receptors (CTR) as well as the ability to form resorption lacunae when the cells are cultured on dentin or bone slices. Udagawa et al showed that spleen cells from mice could be cultured with stromal cell lines or primary osteoblastic cells and induced to form OCL (Udagawa, Takahashi, Akatsu, et al. 1989). The cells formed numerous resorption lacunae and had other characteristics of true osteoclasts. These authors also demonstrated that cell contact between hematopoietic osteoclast precursors in the spleen cell population and stromal or osteoblastic precursors was essential for osteoclast formation.

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2. CHARACTERIZATION OF OSTEOCLAST PRECURSOR CELLS

The osteoclast precursor has been studied to characterize its lineage. Initial work demonstrated that osteoclasts share many characteristics of macrophages (Athanasou, Heryet, Quinn, et al. 1986). Although, osteoclasts and macrophages appear to express some common antigens (Sminia and Dijkstra 1986), there are clear differences in the expression of surface antigens, which separate these two cell types (Kukita and Roodman 1989, Tsurukai, Takahashi, Jimi, et al. 1998). Mononuclear cells that can differentiate into OCL in a variety of in vitro culture systems are present in the bone marrow and the peripheral blood (Quinn, Sabokbar and Athanasou 1996, Fujikawa, Quinn, Sabokbar, et al. 1996).

2.1. Relationship of Osteoclast Precursor Cells to Macrophages

The availability of multiple antibodies recognizing cell surface molecules, which are expressed on hematopoietic cells (Spangrude, Heimfeld and Weissman, et al. 1988, Akashi, Traver, Miyamoto, et al. 2000, Kondo, Weissman, Akashi, et al. 1997, Uchida and Weissman 1992), has allowed the identification of bone marrow and spleen cell populations that can form osteoclast-like cells (OCL) in vitro. Studies from several laboratories have identified several candidate populations with the ability to form OCL in coculture with stromal cells or when cultured alone in liquid media or methylcellulose. In experiments performed before the identification of RANKL, investigators relied on cocultures of various fractions of bone marrow cells with stromal or osteoblastic cells (either primary or cell lines), which were activated to induce osteoclastogenesis by treatment with a stimulator of resorption like 1,25 OH_2 vitamin D. The majority of these early studies focused on monocyte-macrophage lineage cells. Initial studies of cells isolated from bone marrow or spleens showed that cells expressing mature macrophage markers gave rise to OCL when they were cocultured with marrow stromal cells (Cecchini, Hofstetter, Halasy, et al. 1997). Muguruma and Lee identified an osteoclast progenitor population in bone marrow that was negative for mature markers of B-lymphocytes (B220), granulocytes (Gr-1), macrophages (CD11b/Mac-1), and erythroid cells (Ter-119). This population did not express Sca-1, which is a marker that is found on hematopoietic stem cells (HSC) but was positive for the progenitor marker c-kit/CD117 (Muguruma and Lee 1998). These cells could progress to TRAP-expressing mononuclear cells when they were cultured in semi-solid media and OCL cells when they were cultured with vitamin D-treated-ST2 stromal cells. However, the cells in this fraction were considerably multipotential, since they were also able to differentiate into granulocytes, macrophages and erythroid cells. Interestingly, when the c-kit low population was separated, it also could generate osteoclasts, but in a more restricted fashion. Tsurukai et al. isolated cells from coculture of hematopoietic cells and osteoblastic cells by separation through a Sephadex column and found the population of osteoclast precursors to express monocytic markers but not markers of B or T-lymphocytes (Tsurukai, Takahashi, Jimi, et al. 1998). Using a coculture assay with ST2 cells, Hayashi et al found, that osteoclast precursors were in the c-Kit positive fraction and furthermore, that expression of c-fms, the m-CSF receptor, inhibited the efficiency that c-Kit positive cells formed OCL in culture (Hayashi, Miyamoto, Yamane, et al. 1997).

Arai et al. used both coculture with ST2 stromal cells and direct stimulation with RANKL and M-CSF as well as antibodies against the M-CSF receptor, c-fms, and the monocytic marker CD11b/Mac-1, to demonstrate that bone marrow cell populations expressing c-kit formed OCL in culture (Arai, Miyamoto, Ohneda, et al. 1999). These authors concluded that a population of murine bone marrow cells with the phenotype c-kit$^+$ c-fms$^+$ CD11b^{lo}, contained a multipotential progenitor cell population that gave rise to osteoclasts with high frequency. This population did not express RANK when it was isolated from bone marrow. However,

when it was cultured with M-CSF it produced a population of cells that expressed RANK. Interestingly, these precursors were not completely restricted to osteoclastogenesis since in methylcellulose cultures they generated macrophages and mononuclear TRAP positive cells. Microglia, which are specialized phagocytic cells in the central nervous system, appear to also arise from a precursor cell that can give rise to osteoclasts (Servet-Delprat, Arnaud, Jurdic, et al. 2002).

2.2. Relationship of Osteoclast Precursor Cells to Dendritic Cells

The relationship of osteoclasts to dendritic cells, which present antigen to T-lymphocytes as part of the adaptive immune response (Reis e Sousa 2006), is now also established. Cells expressing early markers of myeloid dendritic cell lineage commitment can differentiate into osteoclasts in culture (Servet-Delprat, Arnaud, Jurdic, et al. 2002, Rivollier, Mazzorana, Tebib, et al. 2004, Miyamoto, Ohneda, Arai, et al. 2001). In addition, it appears that relatively mature dendritic cells that can present antigen to T-lymphocytes retain their ability to form osteoclasts, at least, in vitro (Alnaeeli, Penninger, and Teng 2006). The ability of a common progenitor cell to differentiate into macrophages, osteoclasts and myeloid dendritic cells has been proposed for some time (Miyamoto, Ohneda, Arai, et al. 2001, Rivollier, Mazzorana, Tebib, et al. 2004). However, only recently has the identity of a cell that has the capacity to differentiate into macrophages and dendritic cells been demonstrated (Fogg, Sibon, Miled, et al. 2006). It is currently unknown, but highly likely, that the macrophage/myeloid dendritic cell precursor can also differentiate into an OCL.

Expression of the myeloid specific antigen CD11b (Mac-1) has been used by a number of investigators to identify a circulating osteoclast precursor cell (De, Carpentier, Lories, et al. 2004, Yao, Li, Zhang, et al. 2006, Li, Schwarz, O'Keefe, et al. 2004a, Li, Schwarz, O'Keefe, et al. 2004b). The number of these cells in circulation is regulated by the inflammatory state of the organism and in particular tumor necrosis factor. Most recently Yao et al demonstrated that expression of CD11b and Gr1 could be used to identify the levels of this osteoclast precursor in the circulation and the periphery (Yao, Li, Zhang, et al. 2006). We have found that the osteoclast precursor cell in bone marrow is initially negative for expression of CD11b but becomes positive after stimulation with M-CSF and that mature OCL are negative for this antigen (Jacquin, Gran, Lee, et al. 2006). The more important question, which is currently unanswered, is what is the role of the circulating osteoclast precursor cell in normal and pathologic bone turnover? It is currently unknown if this cell homes to bone to form osteoclasts in all conditions where resorption is occurring or if its role is restricted to the development of bone resorption at sites in bone that are not adjacent to marrow. The extramedullary maturation of B and T-lymphocytes is well established (Picker and Siegelman 1999). However, whether similar events occur during osteoclast development is unknown.

3. WHY DO OSTEOCLASTS ONLY FORM IN BONE?

One interesting aspect of osteoclastogenesis is that cells with a cell surface phenotype that is similar to that of osteoclast precursor cells in bone marrow can be identified in the spleen, an organ that also has sources of M-CSF and RANKL. However, osteoclastogenesis does not occur in the spleen under any known condition. One possible explanation for this paradox is that the population of cells found in the spleen, despite having a similar phenotypes to cells found in the bone marrow, are missing crucial elements, which prevent them from forming osteoclasts in splenic tissues. However, this hypothesis seems improbable since multiple investigators have established the in vitro osteoclastogenic potential of splenocytes. Another possibility is that the microenvironment

in the spleen does not allow the production of osteoclasts either because it lacks critical signaling molecules or because it produces inhibitory signals. Miyamoto et al. proposed that in order to complete osteoclastogenesis an adherent condition, which is defined by the expression of specific molecules on osteoclast progenitors, is required (Miyamoto, Arai, Ohneda, et al. 2000). This would ensure the correct interactions between osteoclast progenitors and supporting cells that express the correspondent ligands. Osteoblastic lineage cells in the bone marrow might produce these signals. The absence of these signals in the spleen and other peripheral tissues would explain the inability of these tissues to support osteoclastogenesis.

The latter hypothesis is supported by the recent findings that late osteoclast differentiation and activation require a novel combination of co-stimulatory molecules, which act in concert with M-CSF and RANKL to complete osteoclastogenesis (Colonna 2003). These molecules involve proteins containing immunoreceptor tyrosine-based activation motif (ITAM) domains, which are found in adapter molecules like DAP12 and the Fc receptorγ (FcRγ). The search for receptors associated with these ITAM adaptors in myeloid cells have identified at least two candidates that associate with FcRγ (OSCAR or osteoclast associated receptor, and PIR-A) and two that associate to DAP12 (the triggering receptor expressed by myeloid cells-2, TREM-2), and the signal regulatory protein β1 (SIRP β1) (Koga, Inui, Inoue, et al. 2004). The ligands for these receptors are currently unknown.

4. CAN B-LYMPHOCYTES LINEAGE CELLS FORM OSTEOCLASTS?

Although the myeloid origin of osteoclast is well established, it has been proposed that cells of the B-lymphoid lineage can also give rise to osteoclast progenitors. Several groups have proposed the existence of bipotential progenitors for B lymphocytes and macrophages in bone marrow, which have the ability to differentiate into osteoclasts (Manabe, Kawaguchi, Chikuda, et al. 2001, Blin-Wakkach, Wakkach, Rochet, et al. 2004, Sato, Shibata, Ikeda, et al. 2001). We have also found that Pax-5−/− mice, which have a block in B lymphocyte development at the pro-B cell stage, have an increased number of osteoclasts in their bones and decreased bone mass (Horowitz, Xi, Pflugh, et al. 2004). However, the osteoclast population in these mice appears to be myeloid in origin. In previous work (Katavic, Grcevic, Lee, et al. 2003), we found that OCL formed in cultures of murine bone marrow cells that express the B-lymphocyte marker CD45R/B220. These studies relied on populations of CD45R positive murine bone marrow cells that were separated by fluorescence activated cell sorting (FACS) to a purity of 98–99%. However, in more recent work (Jacquin, Gran, Lee, et al. 2006), we determined that purification of the CD45R/B220 positive murine bone marrow population by a second round of FACS to a purity >99.9% essentially eliminated the ability of the purified CD45R population to form OCL in vitro. Hence, it appears that the OCL, which form in these cultures, require the presence of a contaminating non-CD45R -expressing population of cells to form OCL. We are aware of no other studies of the osteoclastic potential of CD45R -expressing murine bone marrow cells that purified their populations to the degree that we have now done. However, we suspect that additional rounds of purification of other CD45R positive populations, which have been proposed to contain osteoclast precursors, will likely also demonstrate them to be contaminated with small amounts of non-CD45R -expressing osteoclast precursors, which are critical for OCL formation in these cultures.

5. OSTEOCLAST PRECURSOR CELLS IN MURINE BONE MARROW

We have found that osteoclasts precursor cells are enriched in a population of murine bone marrow cells that do not express the antigens CD45R or CD3 and were low for expression CD11b (Jacquin, Gran, Lee, et al. 2006). Culture of these cells with M-CSF and RANKL stimulates

formation of multinucleated TRAP positive osteoclast-like cells (OCL) with high efficiency in as little as 3 days. Interestingly, we also found that murine bone marrow cells, expressing high levels of CD11b, formed osteoclasts with less efficiency than did murine bone marrow cells, which did not express this antigen. OCL in both the CD11b negative/low, (CD45R negative and CD3 negative) and the CD11b high, (CD45R negative and CD3 negative) populations have a number of characteristics of authentic osteoclasts, including the ability to form resorption pits when cultured on bovine cortical bone and high level expression of calcitonin receptors. The highest efficiency of OCL generation was found in the population of murine bone marrow cells that were C-kit and C-fms positive, CD11b negative/low as well as CD45R and CD3 negative (Jacquin, Gran, Lee, et al. 2006).

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CHAPTER 11
INTERACTION WITH ESTROGEN RECEPTORS AS TREATMENT OF ARTHRITIS AND OSTEOPOROSIS

Hans Carlsten

1. INTRODUCTION

Estrogen is a steroid hormone having, in addition to its effects on sexual differentiation and reproduction, important impact on the immune system and on bone. Estrogen exerts its effects via activation of its two receptors ERα and ERβ. Our knowledge of how estrogen manages to mediate its various properties in different organs has increased tremendously by studies of ER targeted mice (Matthews and Gustafsson 2003; Hewitt, Harrell and Korach 2005). It has been evident that the effects of estrogen on bone to a large extent are mediated via its action on immune cells. Estrogen has a dichotomous impact on the immune system by down regulation of inflammatory immune responses but simultaneous upregulation of immunoglobulin production. Consequently, immune mediated diseases in humans and in animal models are modulated by estrogen. Estrogen deficiency after ovarectomy in mice and menopause in women are associated with significant bone loss. In rheumatic diseases such as rheumatoid arthritis (RA) osteoporosis is frequent and in postmenopausal RA the degree of bone loss is dramatically increased. Hormone replacement therapy (HRT) in murine and human arthritis has, as expected beneficial effects on bone loss but it also ameliorates inflammation and inflammation triggered joint destruction (Holmdahl, Jansson, and Andersson 1986; Forsblad d'Elia, Larsen, Mattsson, et al. 2003b). RA is a chronic autoimmune disease with complex pathogenesis (Holmdahl, Bockermann, Backlund, et al. 2002) displaying a variety of inflammatory mechanisms resulting in joint destruction (Gravallese 2002) and generalized osteoporosis (Haugeberg, Orstavik and Kvien 2003). Hence, in order to understand the mechanisms for estrogen mediated effects on bone loss in inflammatory diseases it is necessary to dissect the different stages at which the estrogen has potential modulating properties. Long-term use of HRT has been associated with increased risk of breast cancer, thrombosis and possibly also stroke (Prelevic, Kocjan, and Markou 2005). Accordingly, there is great need for new activators of estrogen receptors (ERs) selectively reproducing only the beneficial effects of estrogen.

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2. ESTROGEN AND THE IMMUNE SYSTEM

A vast majority of patients with autoimmune diseases are women indicating a role for sex steroids. Many studies have revealed that estrogen has the capacity to modulate the course and severity of many chronic neurological and rheumatic diseases (Holmdahl, Carlsten, Jansson, et al. 1989; Grossman, Roselle, and Mendenhall 1991). The knowledge as to the cellular and molecular action of sex steroids has increased and indeed today we can better understand some of the mechanisms whereby estrogen modulates immune responses (Bouman, Heineman, and Faas 2005). We have proposed a dual role of estrogen on immune responses being a suppressor of inflammation but a stimulator of antibody formation. This proposal is based on *in vivo* experiments in mice revealing estrogen as mediator of down regulation of granulocyte induced inflammation (Josefsson, Tarkowski, and Carlsten 1992), T lymphocyte dependent delayed type hypersensitivity (DTH) (Carlsten, Holmdahl, Tarkowski, et al. 1989a,b; Carlsten, Holmdahl, and Tarkowski 1991) and NK cell activity (Hanna and Schneider 1983; Nilsson and Carlsten 1994) on one hand, and up regulation of antigen-specific antibody responses (Carlsten, Holmdahl, Tarkowski, et al. 1989a; Nilsson and Carlsten 1994; Carlsten, Verdrengh, and Taube 1996) as well as polyclonal immunoglobulin production (Nilsson and Carlsten 1994; Erlandsson, Jonsson, Islander, et al. 2002a) on the other. These findings have been repeated by several other groups in different *in vivo* settings (Grossman, Roselle, and Mendenhall 1991). By analyses of estrogen mediated suppression of DTH in several inbred mouse strains and their F1 hybrids we were able to demonstrate that the steroid hormone mediated inhibition of T cell dependent inflammation is genetically linked without association to the MHC complex and inherited as dominant traits (Carlsten, Holmdahl, Tarkowski, et al. 1989a; Carlsten, Holmdahl, and Tarkowski 1991).

Already decades ago it was demonstrated that estrogen in high doses has an impact on the development of the immune system. Sex hormones influence the fetal development of thymus and regulate the size of the thymus gland in adults (Savino and Dardenne 2000). Typically, castration of both sexes leads to an increase of thymus weight and cellularity. Exposure to increased endogenous estrogen levels during pregnancy or treatment of castrated animals with exogenous estradiol causes massive atrophy of the thymus with typically increased frequencies of single $CD4^+$ and $CD8^+$ T lymphocytes. We and others have studied the impact of signaling through ERα and ERβ by analyses of immune organ development and the cellular composition in thymus, spleen and bone marrow of ER targeted mice. These experiments demonstrated that expression of ERα, but not ERβ, is mandatory for development of full-size thymus and spleen, whereas expression of ERβ is required for estradiol-mediated thymic cortex atrophy and phenotype shift of thymic T cells (Staples, Gasiewicz, Fiore, et al. 1999; Erlandsson, Ohlsson, Gustafsson, et al. 2001). Interestingly, in a recent report it was shown that estrogen could induce thymic atrophy also by eliminating early T lymphocytes progenitors already in the bone marrow (Zoller and Kersh 2006).

Estrogen has potent down-regulatory role on B lymphopoiesis in murine bone marrow (Kincade, Medina, and Smithson 1994) but does not dramatically alter other hematopoietic cell progenitors (Thurmond, Murante, Staples, et al. 2000). In intact female mice, B-lymphopoetic cells constitute approximately 10% of all cells in bone marrow whereas after ovarectomy the frequency increases to 15%, to be reduced to 5% in estrogen treated animals (Erlandsson, Jonsson, Lindberg, et al. 2002b; Erlandsson, Jonsson, Islander, et al. 2003). Earlier studies have delineated that a small population of very early B-lineage cells are targets for estrogen mediated regulation of B cell development (Kincade, Medina, Payne, et al. 2000). In more recent experiments we have shown that the inhibitory effect of estrogen on B lymphopoiesis is clearly. ER dependent and that both ERs are required for full reduction of bone marrow B

cells in female and male mice (Erlandsson, Jonsson, Lindberg, et al. 2002; Islander, Erlandsson, Hasseus, et al. 2003). Exposure to estrogen does not only lead to reduction in the number of bone marrow B cells but also to a dramatic shift towards increased differentiation. Interestingly, the frequency of immunoglobulin-switched mature B cells in bone marrow was enriched only in ERα$^+$ mice upon exposure to estrogen. An ERα restricted stimulatory effect on the frequency of immunoglobulin producing cells in both bone marrow and spleen was also seen using the ELISPOT assay (Erlandsson, Jonsson, Lindberg, et al. 2002). A possible mechanism for these phenomena would involve differential expression of ERs in different target cells. Indeed, it has been shown that bone marrow stroma cells express both ERα and ERβ (Smithson, Couse, Lubahn, et al. 1998) while mature B cells in the spleen only express ERα (Benten, Stephan, and Wunderlich 2002).

To summarize, estrogen is a potent inhibitor of the early phase of lymphopoiesis in thymus and in bone marrow but it preserves the more mature lymphocyte phenotypes.

3. ESTROGEN AND ARTHRITIS

It has been clearly demonstrated that endogenous and exogenous estrogen have potent anti-arthritic effects in models of RA such as collagen type II induced arthritis (CIA) (Holmdahl, Carlsten, Jansson, et al. 1989) and that this effect of estrogen is ER-dependent (Jansson and Holmdahl 2001). Endogenous estrogen can exclusively explain the very mild expression of disease in female mice and OVX significantly accelerates arthritis (Jansson and Holmdahl 1989). Generalized osteoporosis in postmenopausal RA is caused both by estrogen deficiency and the inflammatory disease but the relative importance of each of these factors is unknown. We therefore established a murine model of osteoporosis in postmenopausal RA, and evaluated the relative importance of menopause versus arthritis-related osteoporosis (Jochems, Islander, Erlandsson, et al. 2005). To mimic postmenopausal RA, DBA/1 mice were ovariectomized, followed by induction of arthritis using immunization with type II collagen. The known anti-inflammatory effect of estrogen was revealed since OVX mice displayed more severe arthritis compared to sham-operated controls. At sacrifice, arthritic control mice and non-arthritic OVX mice displayed trabecular bone loss of 26% and 22%, respectively. OVX mice with arthritis had as much as 58% decrease of trabecular BMD. Thus, the loss of endogenous estrogen and inflammation contributed equally much to osteoporosis in experimental postmenopausal polyarthritis.

In postmenopausal RA the consequence of osteoporosis i.e. fractures are a major clinical problem. It is not possible to directly quantitate the relative contribution of the inflammatory disease and estrogen deficiency, respectively to osteoporosis. We tested if there is an association between determinants of joint destruction and BMD in postmenopausal women with active RA not treated with bisphosphonates or HRT and if there are common markers of erosive disease and bone loss in these patients. BMD was measured and joint damage was examined by x ray examination according to the Larsen method (Forsblad D'Elia, Larsen, Waltbrand, et al. 2003a). As much as 56% of the patients had osteoporosis in at least one site, most of them (48%) in the femoral neck. The corresponding figure for healthy age-matched women is only 7%, demonstrating that the inflammatory disease and/or its treatment certainly contribute to loss of bone. Linear regression analyses revealed that low body weight and high Larsen score were strongly associated with BMD reduction and that increased CRP and long disease duration were determinants of erosive disease in postmenopausal women with RA. Altogether, these findings reveal that osteoporosis is very common in postmenopausal RA and indicate common mechanisms of local and generalized bone loss in RA (Forsblad D'Elia, Larsen, Waltbrand, et al. 2003a).

We have analyzed not only the effects of exposure to estradiol on the joint disease but also on the course of osteoporosis in CIA mice (Jochems et al. unpublished). Clearly, both prophylactic and therapeutic protocols reveal that administration of exogenous E2 efficiently blocks the inflammatory joint disease as well as the development of osteoporosis. Notably, these experiments also included treatment with raloxifene (SERM) and data unquestionably showed similar therapeutic effects of this compund.

Is estrogen also an efficient compound for treatment of arthritis and osteoporosis in postmenopausal RA? Indeed, HRT is known to exert a positive effect in preventing bone loss but only a few rather small and short studies with conflicting results have analyzed its effect on the disease activity and joint destruction in RA (Hall, Daniels, Huskisson, et al. 1994; MacDonald, Murphy, Capell, et al. 1994). Hence, we evaluated the effects of HRT on BMD and on the course of established RA in a two year long, randomized controlled study. Treatment with HRT significantly suppressed signs of inflammation as shown by reduction of erythrocyte sedimentation rate (ESR) and an elevation of hemoglobin concentration. Further, a better clinical outcome as assessed by response using Disease Activity Score 28 (DAS28) and increased BMD in the forearm, proximal femur and spine was obtained. The most striking finding was that HRT significantly retarded progression of joint destruction among patients with radiologically progressive disease (Forsblad d'Elia, Larsen, Mattson, et al. 2003c). The mechanisms by which HRT exerts these beneficial clinical effects are unclear but we found that HRT for 2 years resulted in an increase of the bone anabolic factor IGF-1 and a decrease of sIL-6R, which is known to enhance the biological activity of IL-6 (Forsblad d'Elia, Mattsson, Ohlsson, et al. 2003d). In a following study it was suggested that biochemical markers of bone and cartilage turnover may provide a useful tool for assessing novel treatment modalities in arthritis, concerning both joint protection and prevention of osteoporosis (Forsblad d'Elia, Christgau, Mattsson, et al. 2004).

Altogether we have shown that HRT has many beneficial effects regarding treatment of erosive arthritis and osteoporosis. Thus we suggest that HRT can be used in addition to conventional therapy in the management of postmenopausal patients with RA. However, the side effects related to long-term use of combination therapy encompassing estrogen and progesterone must be considered in each patient.

4. ESTROGEN AND BONE

Estrogen has besides its role for sexual differentiation and development also important impact on the skeleton of females and males (Compston 2001; Vanderschueren, Vandenput, Boonen, et al. 2004). For instance, the epiphyseal closure at puberty is in both sexes dependent on estrogen and thus the higher stature of men compared to females can be explained by the later peak of estrogen production in boys (Lombardi, Zarrilli, Colao, et al. 2001). Osteoporosis is characterized by reduced bone mass and disruption of bone architecture, leading to increased bone fragility and increased risk for fractures particularly of the hip, spine, and wrist. Already in the 1940s, the central role of estrogen deficiency in the pathogenesis of postmenopausal osteoporosis was recognized. These findings and the subsequent increased knowledge in bone physiology have changed the previous attitude that osteoporosis was an untreatable condition and a consequence of normal ageing. This change in the view of women's health after menopause resulted in a dramatic increase in consumption of estrogen containing HRT during the last four decades. The percentage of postmenopausal women on HRT in the Western World increased to more than 40% by year 2000. The drastic reduction down to today's 20%, corresponding to

levels of the early 1980s was the result of findings in the WHI and "the Million Women Study" demonstrating increased risk of stroke, coronary heart disease and breast cancer associated with long-term treatment with HRT containing combined estrogen and progesterone.

During the last decades several mechanisms for estrogen mediated effects on bone loss have been proposed. It is reasonable to assume that a combination of molecular actions of the hormone are working together both directly on cells in the bone compartment but also indirectly via other biological systems. Lately, cells of the immune system have been recognized as target cells for estrogenic effects on bone.

In clinical studies serum IL-6 levels have been found to predict bone loss in postmenopausal women, and increase of sIL-6R following ovarectomy has been prevented by estrogen treatment. Administration of hormone replacement therapy (HRT) to healthy postmenopausal women decreased serum levels of sIL-6R (Pfeilschifter , Koditz, Pfohl, et al. 2002). In addition, neutralization of IL-6 with antibodies or IL-6 gene silencing in mice prevent a) the up regulation of granulocyte and macrophage proliferation in the bone marrow, b) the expected increase of osteoclast numbers in trabecular bone, c) protect against sex steroid dependent bone loss. The suppressive effect of estrogens and androgens on IL-6 production has been found to be mediated via the classical sex steroid receptors (Bellido, Jilka, Boyce, et al. 1995; Pottratz, Bellido, Mocharla, et al. 1994).

Estrogens also influence other important osteoclast-stimulating cytokines such as M-CSF, IL-1 and TNF-α. It has for instance been shown that the production of IL-1 and IL-1 receptor antagonist (IL-1ra) by peripheral blood monocytes was increased in both oophorectomized (OVX) and postmenopausal women and consequently suppressed by estrogen replacement therapy (Pacifici, Vannice, Rifas, et al. 1993).

In a number of studies Pacifici and coworkers have suggested a model for the cellular and molecular mechanisms responsible for OVX induced bone loss in mice. They showed that OVX of mice failed to induce bone loss, stimulate bone resorption, or increase M-CSF- and RANKL-dependent osteoclastogenesis in T-cell deficient mice, establishing T cells as essential mediators of the bone loss associated to estrogen deficiency *in vivo* (Cenci, Weitzmann, Roggia, et al. 2000). In addition, they found that OVX expanded and prolonged the life-span of a population of TNF-producing T cells in bone marrow and that in TNF-deficient mice OVX failed to induce bone loss, a property restored after transfer of $TNF^{+/+}$ T cells (Roggia, Gao, Cenci, et al. 2001; Cenci, Toraldo, Weitzmann, et al. 2003). These data have very recently been contradicted by the finding that T lymphocyte deficient nude, RAG and TCRαKO mice lost trabecular bone upon ovarectomy (Lee, Kandono, Okada, et al. 2006).

Is estrogen acting directly on bone marrow macrophages or T cells or is it acting through other regulatory systems? Although T-lymphocytes express low density ERs, our earlier studies excluded that estrogen exerts direct effects on T cells when acting as a down regulator of DTH in SCID mice reconstituted with normal T cells (Taube, Svensson, and Carlsten 1998). Instead, we recently suggested that the growth hormone (GH)/insulin like growth factor I (IGF-I) axis could be involved. Interestingly, we found that in mice with adult liver specific IGF-I inactivation (LI-IGF-$I^{-/-}$) OVX did not reduce trabecular bone mineral density. Simultaneously, OVX increased the number of T-cells in the bone marrow of WT but not of LI-IGF-$I^{-/-}$ mice. Interleukin 7 (IL-7) has been reported to stimulate the formation and function of osteoclasts by inducing the expression of receptor activator of NF-κB ligand (RANKL) on T-cells (Miyaura, Onoe, Inada 1997). IL-7 mRNA levels and the RANKL/osteoprotegrin ratio in bone were increased by OVX in WT but not in LI-IGF-$I^{-/-}$ mice. These data demonstrate that liver-derived IGF-I is permissive for OVX-induced trabecular bone loss indicating that IGF-I might exert this permissive action by modulation of T-cell proliferation and the expression of IL-7. The latter

finding is of importance for the RANKL/OPG ratio and consequently osteoclastogenesis in the bone marrow (Lindberg, Svensson, Venken, et al. 2006).

In conclusion, sex hormones influence bone turnover in a complex way and the knowledge about their action increases constantly. Estrogen has been found to decrease the number of remodeling cycles through attenuating the number of osteoblasts and osteoclasts from their respective progenitors, to display pro-apoptotic effects on osteoclasts and anti-apoptotic effects on osteoblasts. Importantly, the cytokines and hormones involved in bone metabolism also influence the immune system and inflammatory processes.

5. ESTROGEN RECEPTOR MODULATION

Estrogen is mainly synthesized from its precursor cholesterol in the ovaries but also produced by aromatization of testosterone in several tissues. The main effects of estrogen are mediated through activation of ERs belonging to the nuclear receptor family. ERα was cloned 1986 and ERβ in 1995. ERs are ligand activated transcription factors with a highly conserved DNA-binding domain and a less conserved ligand-binding domain. In the classical ER signaling pathway ligand activated ERs dimerize and serve as activating transcription factors at gene promotors containing specific estrogen response elements (ERE). Nuclear ER-ERE complexes attract tissue-specific co regulators that either enhance or reduce transactivation. The effects of estrogen on the reproductive functions are regarded to be mediated via this classical pathway directly at cells in the target organ. Transcription can also be affected by a direct binding of ligand-activated ER protein to transcription factors such as specific protein–1 (SP1), activation protein-1 (AP1) and nuclear factor κB (NFκB) proteins. For instance, the suppressive effect of estrogen on the production of IL-6 and TNF-α has been shown to be mediated via down regulation of NFκB and AP-1, respectively (Pfeilschifter, Koditz, Pfohl, et al. 2002; McKay and Cidlowski 1999). In such cases activation of membrane bound or cytoplasmatic receptors have been proposed. In addition to the classical genomic action of estrogen, non-genomic effects have been described (Bjornstrom and Sjoberg 2005). Hence, some effects of estrogen are very rapid making genomic mechanisms unlikely. These non-classical signaling pathways involve both steroid and non-steroid receptors which also can activate kinases that ultimately regulate transcription of specific genes (Lorenzo 2003). Recently, the G protein-coupled receptor 30 (GPR 30) was shown to specifically bind estrogen and this interaction resulted in intracellular calcium mobilization and nuclear synthesis (Revankar, Cimino, Sklar, et al. 2005).

Selective estrogen receptor modulators (SERMs) are synthetic ligands with tissue specific agonistic or antagonistic properties. Tamoxifene was developed as an ER antagonist for treatment of breast cancer but was later on recognized to exert agonistic effects on endometrium and skeleton. Raloxifene was shown to have agonistic effects in bone and antagonistic properties in breast tissue. Finally, ICI 182,780 acts as a peripheral ER antagonist lacking all agonistic effects. We compared the effects of raloxifene and estradiol on T- and B-lymphopoiesis, immune responsiveness and inflammation (Erlandsson, Jonsson, Lindberg, et al. 2002b; Erlandsson, Gomori, Taube, et al. 2000). In OVX mice treated with subcutaneous injections of equipotent anti-osteoporotic doses of raloxifene (3 mg/kg) and estradiol (0.1 mg/kg) we analyzed effects of these substances on thymus phenotypes and peripheral T cell functions. Raloxifene induced only minor thymus atrophy and totally lacked the suppressive properties of estradiol on T cell dependent DTH and granulocyte-mediated inflammation (Erlandsson, Gomori, Taube, et al. 2000). By contrast, it exerted full inhibition of B-lymphopoiesis and triggered partially bone marrow resident immunoglobulin-producing B cells (Erlandsson, Jonsson, Lindberg, et al. 2002b).

The isoflavone genistein is a naturally occurring phytoestrogen found in high concentrations in soy products. Genistein binds ERs and induces mainly ERβ mediated transcription and it is also a potent protein tyrosine kinase (PKT) inhibitor that affects cell cycling and smooth muscle contraction. Some studies have demonstrated effects of genistein on osteoporosis, certain types of cancers, bacterial growth and cardiovascular diseases (Chen and Rogan 2004), potent inhibitory effects on thymus (Yellayi, Naaz, Szewczykowski, et al. 2002) and suppressed B-lymphopoiesis (Ishimi, Arai, Wang, et al. 2000). In a recently published study (Erlandsson, Islander, Moverare, et al. 2005), we were unable to repeat above results. Instead, we demonstrated that genistein had antagonistic effects on uterus weight and on B-lymphopoiesis in sham operated female mice and weak agonistic effects on bone mineral density in OVX mice.

A few years ago it was (Kousteni, Bellido, Plotkin, et al. 2001) proposed that sex steroids affect bone through non-genomic pathways. It was demonstarted that (Kousteni, Chen, Bellido, et al. 2002) treatment of mice with a synthetic estrogen-like compound, 4-estren-3α,17β-diol, (estren) increases bone mass in OVX animals without affecting the rep t reproduces only the non-genomic signaling of estrogen and thus can also affect target cells through the AR (Kousteni, Han, Chen, et al. 2003). However, using ER double knock-out mice we recently demonstrated that estren has moderate uteroproliferative effect and that the trabecular bone sparing effect of estren *in vivo* in females is mediated via ERs and not via the AR (Moverare, Dahllund, Andersson, et al. 2003). In following experiments it was revealed that estren suppresses both T- and B-lymphopoiesis via ER independent and possibly AR dependent mechanisms while it suppresses T cell dependent inflammation, IL-6 production and frequency of splenic $CD4^+$ T cells through ER dependent mechanisms (Islander, Erlandsson, Chavoshi, et al. 2005; Islander, Hasseus, Erlandsson, et al. 2005).

6. CONCLUSIONS

Estrogen has strong impact on the development and regulation of the immune system and on bone remodelling. There is no dout that HRT has beneficial effects on arthritis and osteoporosis in postmenopausal RA. However, since long-term treatment with estrogen and progesterone increases the risk for breast cancer this type of therpy is no longer recommended. Hence, there is need to increases our knowledge regarding the cellular and molecular action of estrogen and synthetic ER modulators in order to find an efficacious and safe remedy for postmenopausal patients with chronic arthritis and osteoporosis.

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CHAPTER 12
NOVEL SIGNALING PATHWAYS AND THERAPEUTIC TARGETS IN OSTEOCLASTS

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1. INTRODUCTION

Osteoclasts are multinucleated cells of monocyte/macrophage origin that degrade bone matrix. Their differentiation is mainly dependent on a TNF family cytokine, receptor activator of nuclear factor (NF)-κB ligand (RANKL), and is also under extensive control by a variety of immunomodulatory molecules (Theill, Boyle, and Penninger 2002; Takayanagi 2005; Sato, and Takayanagi 2006). Therefore, osteoclast biology has been in the forefront of osteoimmunology since the very beginning (Arron and Choi 2000; Takayanagi, et al. 2000b). Congenital lack of osteoclasts causes osteopetrosis, which provided insights into essential molecules for osteoclastogenesis including TRAF6, NF-κB and c-Fos (Teitelbaum, and Ross 2003). In addition, genomewide screening techniques shed light on an hitherto unknown set of genes such as nuclear factor of activated T cells (NFAT) c1 (Takayanagi, et al. 2002a). It is of great importance to understand the sequential molecular events mediated by such essential molecules (TRAF6, NF-κB, c-Fos and NFATc1) in the context of RANKL signaling during osteoclast differentiation. Furthermore, an increasing number of molecules are known to regulate RANKL-mediated osteoclast differentiation. These studies do have not only scientific but also clinical significance, providing novel therapeutic targets in osteoimmunological diseases.

2. OSTEOCLAST AND RANKL IN THE FOREFRONT OF OSTEOIMMUNOLOGY

The immune and skeletal systems share a number of regulatory molecules including cytokines, receptors, signaling molecules and transcription factors (Walsh, et al. 2006). Furthermore, immune cells are formed in the bone marrow, interacting with bone cells. Therefore, it is reasonable to consider that the physiology and pathology of one system may affect the other. Notably, an

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abnormal or prolonged activation of the immune system leads to bone destruction in autoimmune diseases such as rheumatoid arthritis (Takayanagi, et al. 2000a). Bone homeostasis depends on the balanced action of bone-forming osteoblasts and bone-resorbing osteoclasts (Karsenty and Wagner 2002; Teitelbaum, and Ross 2003). RANKL is a tumor necrosis factor (TNF) family cytokine, which was identified to be an essential factor for the induction of osteoclastogenesis (Lacey, et al. 1998; Yasuda, et al. 1998). However, RANKL is also expressed by activated T cells, suggesting that this molecule is important in both the skeletal and immune systems (Anderson, et al. 1997; Wong, et al. 1997). Indeed, the targeted disruption of RANKL results in the defective formation of lymph nodes and lymphocyte differentiation, as well as osteopetrosis, a sclerotic bone disease caused by impaired osteoclastic bone resorption (Kong, et al. 1999).

We revealed the molecular mechanism of T-cell-mediated regulation of osteoclast formation through a signaling crosstalk between RANKL and interferon (IFN)-γ (Takayanagi, et al. 2000). In a commentary article in Nature, Arron and Choi coined a word, "osteoimmunology" to describe this new interdisciplinary field of bone biology and immunology (Arron, and Choi 2000). However, RANKL is not the only factor linking the immune and skeletal systems. A targeted disruption of immunomodulatory molecules results in an unexpected phenotype in the skeletal system, leading us to explore the regulatory mechanism of bone metabolism by the immune system (Takayanagi, et al. 2002b; Yagi, et al. 2005; Jones, et al. 2006) Thus, osteoimmunology is becoming increasingly important for understanding the pathogenesis and developing new therapeutic strategies for diseases affecting both systems.

3. TRANSCRIPTIONAL REGULATION BY NFATc1

RANKL signaling stimulates the activation of NF-κB, AP-1 and MAPKs, but similar pathways are activated by cytokines such as IL-1 that do not induce osteoclastogenesis. Therefore, it was suggested that RANKL has unknown specific target(s) to induce terminal differentiation of osteoclasts. Genome-wide screening techniques contributed greatly to clarify one of the key targets: nuclear factor of activated T cells c1 (NFATc1) was shown to be strongly induced by RANKL (Takayanagi, et al. 2002a). NFATc1 expression is dependent on TRAF6 and c-Fos pathways activated by RANKL, suggesting an integral role of NFATc1 in RANKL signaling. Activation of NFAT is mediated by a phosphatase, calcineurin, which is activated by calcium/calmodulin signaling. Consistent with this, calcineurin inhibitors such as FK506 and cyclosporin A strongly inhibit osteoclastogenesis. The essential and sufficient role of the *NFATc1* gene in osteoclastogenesis was suggested by the *in vitro* observation that *NFATc1*$^{-/-}$ embryonic stem cells do not differentiate into osteoclasts and that the ectopic expression of NFATc1 causes the bone marrow precursor cells to undergo osteoclast differentiation in the absence of RANKL (Takayanagi, et al. 2002a). Importantly, *in vivo* evidence for the role of NFATc1 has been provided recently. The analysis of NFATc1-deficient mice has been hampered by the embryonic lethality, but the adoptive transfer of hematopoietic stem cells to osteoclast-deficient c-*fos*$^{-/-}$ mice made it possible to show the critical function of NFATc1 in osteoclastogenesis *in vivo* (Asagiri, et al. 2005).

Taken together the previous reports, the mechanism of RANKL-induced osteoclastogenesis can be summarized as follows. RANKL bind to its receptor RANK, which recruits adaptor molecules such as TRAF6. TRAF6 activates NF-κB, which is important for the initial induction of NFATc1. NFATc1 is activated by calcium signaling and binds to its own promoter, thus switching on the autoregulatory loop. An AP-1 complex containing c-Fos is required for the autoamplification of NFATc1, enabling the robust induction of NFATc1. Finally, NFATc1 cooperates with other transcriptional partners to activate osteoclast-specific genes. Interestingly, NFATc1 autoregulation is controlled by an epigenetic mechanism.

4. CRITICAL ROLE OF ITAM SIGNALING FOR NFATc1 INDUCTION

The immunoreceptor tyrosine-based activation motif (ITAM) signaling mediated by dual membrane adaptors, Fc receptor (FcR) common γ subunit (FcRγ) and DNAX activating protein 12 (DAP12), is essential for RANKL induction of osteoclast differentiation based on the analysis of *DAP12*$^{-/-}$*FcRγ*$^{-/-}$ mice (Koga, et al. 2004). FcRγ and DAP12 associate with multiple immunoreceptors such as OSCAR and TREM-2, which activate costimulatory signals for RANK. The induction of NFATc1 is severely suppressed in *DAP12*$^{-/-}$*FcRγ*$^{-/-}$ cells. The ectopic expression of NFATc1, not c-Fos or TRAF6, resulted in efficient osteoclast formation even in *DAP12*$^{-/-}$*FcRγ*$^{-/-}$ cells, suggesting that NFATc1 induction is the critical downstream event of ITAM signaling. Furthermore, calcium oscillation induced by RANKL (Takayanagi, et al. 2002a) is not significant in *DAP12*$^{-/-}$*FcRγ*$^{-/-}$ cells. These results suggest that FcRγ and DAP12 are required for the induction of NFATc1, the critical step in the RANKL-induced osteoclast differentiation program, through the activation of calcium signaling. RANKL-induced phosphorylation of PLCγ is impaired in *DAP12*$^{-/-}$*FcRγ*$^{-/-}$ cells. It is also important that osteoclast differentiation is inhibited by Syk inhibitors (Koga, et al. 2004) and is impaired in Syk-deficient cells (Faccio, Zou, Colaianni, et al. 2003; Mocsai et al. 2004). In addition, RANKL induces the phosphorylation of both FcRγ and DAP12, an event linking the RANKL and ITAM signaling. Taken together, the phosphorylation of ITAM stimulated by immunoreceptors and RANKL-RANK interaction results in the recruitment of Syk family kinases, leading to the activation of PLCγ and calcium signaling, which is critical for NFATc1 induction.

The importance of the ITAM-harboring adaptors and the receptors associated with them in bone metabolism is also underscored by the reports that mutations in *DAP12* and *TREM-2* genes cause skeletal and psychotic abnormalities called Nasu–Hakola disease (Paloneva, et al. 2002, 2003; Cella, et al. 2003).

5. SUMMARY AND FUTURE DIRECTIONS

The discovery of the RANKL-RANK system and the identification of NFATc1 as the master regulator of osteoclastogenesis brought us a rapid progress in the understanding of the regulatory mechanism of osteoclast differentiation. The importance of calcium-calcineurin signaling in osteoclast differentiation is also appreciated and provided insights into the role of ITAM and other calcium-related signaling molecules in the differentiation of osteoclasts. We believe that these studies will provide unprecedented therapeutic strategies for skeletal and immune disorders in the future.

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CHAPTER 13
THE ENIGMATIC FUNCTION OF TREM-2 IN OSTEOCLASTOGENESIS

Marco Colonna, Isaiah Turnbull, and Julia Klesney-Tait

1. ABSTRACT

The triggering receptor expressed on myeloid cells 2 (TREM-2) is a member of family of receptors that play a central role in regulating function of myeloid cells. TREM-2 is expressed on macrophages, microglia and pre-osteoclasts and transduces intracellular signals through the adaptor DAP12. In human, genetic defects of TREM-2 and DAP12 result in a rare syndrome characterized by presenile dementia and bone cysts. This syndrome and the tissue distribution of TREM-2 have indicated a role of the TREM-2/DAP12 complex in brain function and bone modeling, particularly osteoclastogenesis. Accordingly, human TREM-2- and DAP12-deficient pre-osteoclast precursors failed to differentiate *in vitro* into mature osteoclasts endowed with bone resorptive activity. In mouse, DAP12-deficiency also resulted in impaired osteoclastogenesis *in vitro* and a mild osteopetrosis *in vivo* although bone cysts were not observed. Surprisingly, TREM-2-deficiency in mouse led to accelerated osteoclastogenesis *in vitro* without osteopetrosis or bone cysts *in vivo*, revealing an unexpected inhibitory function of mouse TREM-2. These data demonstrate that TREM-2 function is essential for normal osteoclastogenesis. The conflicting results as to the relationship between TREM-2, DAP12 and osteoclastogenesis and bone modeling in human and mouse suggest that TREM-2 contribution to osteoclast biology may vary depending on the influence of additional DAP12-associated receptors and on the presence of TREM-2 ligands with variable avidity/affinity, which may induce either activating or an inhibitory signals through TREM-2/DAP12.

2. THE ITAM SIGNALING PATHWAY IN OSTEOCLASTOGENESIS

Osteoclasts are multinucleated giant cells of myeloid origin involved in bone resorption and homeostasis (Teitelbaum 2000). Development of osteoclasts from myeloid progenitors involves multiple signaling pathways. The M-CSF/M-CSF-R and RANK/RANKL pathways

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have been extensively characterized and shown to be essential in osteoclastogenesis (Takayanagi 2005; Teitelbaum 2000; Walsh, Kim, Kadono, et al. 2006). More recently, attention has focused on the pathways triggered by cell surface receptors that signal through the DNAX-activating protein 12 (DAP12, also called KARAP) and Fc receptor-common gamma chain (FcRγ) (Humphrey, Lanier, and Nakamura 2005; Koga, Inui, Inoue, et al. 2004; Mocsai, Humphrey, Van Ziffle, et al. 2004).

DAP12 and FcRγ are transmembrane adaptors expressed as disulfide bonded homodimers. They associate with a variety of cell-surface receptors via complementary charged transmembrane domains that form a salt-bridge in the context of the hydrophobic lipid bilayer. DAP12 and FcRγ cytoplasmic domains contain immunoreceptor tyrosine-based activation motif (ITAM) (Lanier 2003; McVicar and Burshtyn 2001; Nadler, Matthews, Turner, et al. 2000; Vivier, Nunes, and Vely 2004). In response to receptor ligation, Src kinases phosphorylate the tyrosines in the ITAM, forming docking site for the protein tyrosine kinases Syk and ZAP70. Syk and ZAP70 then phosphorylate the scaffolding molecules LAT and NTAL, recruiting proximal signaling molecules phosphatidylinositol 3-kinase (PI3-K), phospholipase C gamma, the SLP76/Vav complex, the Grb2/Sos complex and c-Cbl. These activate downstream signaling cascades resulting in intracellular Ca^{2+} mobilization, activation of PKC, activation of MAP-kinases and rearrangement of the actin cytoskeleton.

The importance of the ITAM signaling pathway in osteoclastogenesis has been recently demonstrated (Koga, Inui, Inoue, et al. 2004; Mocsai, Humphrey, Van Ziffle et al. 2004). In these studies, mice lacking both DAP12 and FcRγ exhibited impaired osteoclast differentiation, leading to severe osteopetrosis. Specifically, DAP12 and FcRγ are required for the generation of calcium signals that lead to induction of nuclear factor of activated T cells c1 (NFATc1), which is a key transcription factor for osteoclastogenesis (Asagiri, Sato, Usami, et al. 2005). Thus, although M-CSF/M-CSF-R and RANKL/RANK pathways are necessary for osteoclastogenesis, they are not sufficient, as the ITAM pathway is also required to activate osteoclastogenesis. In this article, we will focus on the function of one DAP12-associated receptor, the triggering receptor expressed on myeloid cells 2 (TREM-2), which has been recently shown to play a critical role in osteoclastogenesis.

3. THE TREM-2/DAP12 COMPLEX

DAP12 and FcRγ associate with multiple receptors, which are expressed inside and outside the immune system, including natural killer (NK) cells, B cells, monocytes, macrophages, dendritic cells, microglia and osteoclasts (Lanier 2003; McVicar and Burshtyn 2001; Nadler, Matthews, Turner, et al. 2000; Vivier, Nunes, and Vely, et al. 2004). We have concentrated our attention on a group of DAP12-associated receptors called triggering receptors expressed on myeloid cells (TREM). TREM receptors are cell surface glycoproteins encoded by a cluster of genes on human chromosome 6p21 and mouse chromosome 17C3 (Allcock, Barrow, Forbes, et al. 2003; Klesney-Tait, Turnbull, and Colonna 2006). TREM receptors include TREM-1, TREM-2, and in the mouse, TREM-3, as well as at least two other TREM-related molecules, called TREM-like Transcript (TLT)-1 and TLT-2. All of these receptors are members of the immunoglobulin super family and contain a single V type Ig domain. The closest TREM relative is NKp44, an activating NK cell receptor encoded by a gene closely linked to the TREM gene cluster (Cantoni, Bottino, Vitale, et al. 1999). More distant relatives of TREMs include CD300 family members, as well as the polymeric Ig receptor (Aguilar, Alvarez-Errico, Garcia-Montero, et al. 2004; Chung, Humphrey, Nakamura, et al. 2003; Jackson, Hart, Starling, et al. 1992).

Among TREM receptors, TREM-1 and TREM-2 have been more extensively characterized. The first TREM identified, TREM-1, is an amplifier of the inflammatory response that strongly potentiates the activation of granulocytes and macrophages to microbial products. The function

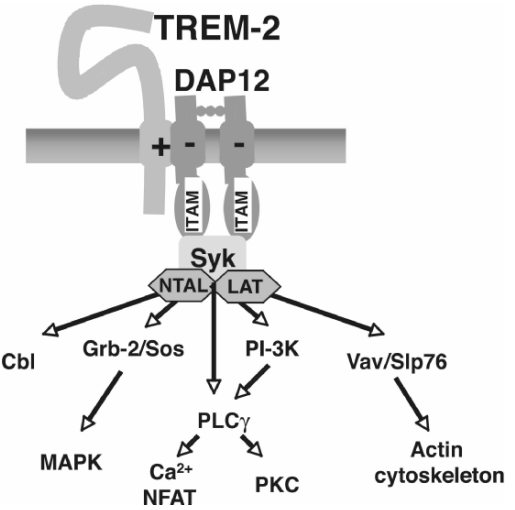

Figure 1. TREM-2/DAP12 signaling. TREM-2 associates with DAP12 for cell surface expression and signaling. Engagement of TREM-2 leads to tyrosine phosphorylation of DAP12 ITAM and subsequent recruitment of Syk. Syk promotes phosphorylation and recruitment of the signaling adaptors NTAL and LAT which trigger a signaling cascade leading to Ca2+ mobilization, PKC and MAP kinase activation and rearrangement of actin cytoskeleton (*See Color Plate*).

of TREM-1 has been recently reviewed (Klesney-Tait, Turnbull, and Colonna 2006). Because of its involvement in osteoclastogenesis, here we will focus on TREM-2. TREM-2 consists of a single V type Ig ectodomain, a transmembrane region and a short cytoplasmic tail lacking any signaling motifs. The transmembrane domain of TREM-2 has a positively charged residue, which mediates formation of a com plex with DAP12 (Figure 1). DAP12 is essential for surface expression and signaling by TREM-2.

TREM-2 was originally identified in human monocyte derived DC and shown to promote DC migration through the upregulation of the chemokine receptor CCR7 (Bouchon, Hernandez-Munain, Cella, et al. 2001). However TREM-2 has not been detected in primary DC and therefore it role in DC biology remains unclear. TREM-2 expression has been also demonstrated in macrophage cell lines (Daws, Lanier, Seaman, et al. 2001), bone marrow derived macrophages (Humphrey, Daws, Spusta, et al. 2006), macrophages recruited to the peritoneum following treatment with thioglycollate and on alternatively activated macrophages (Turnbull, Gilfillan, Cella, et al. 2006), suggesting a role for TREM-2 in macrophage biology. Beyond DC and macrophages, TREM-2 expression has been reported outside the immune system, in pre-osteoclasts (Cella, Buonsanti, Strader, et al. 2003; Paloneva Manninen, Christman, et al. 2002) and microglia (Schmid, Sautkulis, Danielson, et al. 2002; Sessa, Podini, Mariani, et al. 2004), suggesting a function for TREM-2 in bone and brain biology. Indeed, such as role has been demonstrated by recent studies of a rare genetic disorder, called Nasu-Hakola disease (NHD).

4. THE NASU-HAKOLA DISEASE

NHD, also called polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL), is a recessively inherited disorder characterized by systemic bone cysts and progressive presenile dementia associated with sclerosing encephalopathy (Hakola, Jarvi, and Sourander 1970; Nasu, Tsukahara, and Terayama 1973; Paloneva, Autti, Raininko, et al. 2001).

The disease has been initially reported in Finland and in Japan, but has a worldwide distribution, with cases reported in South America and Italy. NHD patients develop joint swelling and/or fractures following minor accidents between the ages of 20–30. Multiple bone cysts are visible on x-ray and (in the absence of neoplasia) are pathognomic of Nasu-Hakola disease in affected populations. More insidious than the bone pathology, NHD patients develop a presenile dementia that is progressive and ultimately fatal. The central nervous system (CNS) of NHD patients contains sclerotic lesions including activated microglial cells particularly affecting the white matter of the brain.

The original study of Finnish NHD reported linkage of the disease to a region on chromosome 19. Analysis of the potential candidate genes in this chromosomal region revealed a homozygous deletion of exons 1–4 of the DAP12 gene (Paloneva, Kestila, Wu, et al. 2000). Confirming the role of DAP12, several Japanese and South American NHD cases were found to have a loss-of-function DAP12 mutation (Bianchin, Capella, Chaves, et al. 2004; Kondo, Takahashi, Kohara, et al. 2002). Subsequent analysis of NHD patients with intact DAP12 genes and normal levels of DAP12 expression revealed loss of function mutations in TREM-2 (Paloneva, Manninen, Christman, et al. 2002; Soragna, Papi, Ratti, et al. 2003). TREM-2- and DAP12-deficient patients develop identical disease, suggesting that the clinical manifestations of bone cysts and CNS disease result from the failure of TREM-2 signaling, either due to a defect in the TREM-2 receptor itself or in the associated DAP12 signaling chain. Given that TREM-2 is expressed in pre-osteoclasts and microglia, it is likely that the defects in the bone and the CNS observed in NHD patients result from a functional failure of these cells in the bone and brain respectively.

5. TREM-2 IS REQUIRED FOR OSTEOCLASTOGENESIS

To determine the potential role of the TREM-2/DAP12 complex in osteoclast function, studies were performed in human TREM-2- and DAP12-deficient osteoclasts in vitro and in DAP12-deficient mice both in vitro and in vivo. Osteoclasts can be derived in-vitro from blood monocytes or bone marrow cells by culture with M-CSF and RANKL, leading to fusion and differentiation of cultured cells into multinucleate osteoclasts capable of resorbing bone in vitro. In these culture conditions, blood monocyte derived from TREM-2- or DAP12-deficient patients were incapable of fusing into multinucleated osteoclasts endowed with bone resorptive activity (Table 1) (Cella, Buonsanti, Strader, et al. 2003; Paloneva, Mandelin, Kiialainen, et al. 2003). These data were validated using DAP12−/− mice. Murine DAP12−/− bone marrow cells failed to form bone-resorbing osteoclasts in culture, consistent with the human phenotype (Table 1) (Kaifu, Nakahara, Inui, et al. 2003). Similar conclusions were reached by a recent study by Humphery et al. in which TREM-2 expression in osteoclast precursors is attenuated by siRNA (Humphrey, Daws, Spusta, et al. 2006). These authors found that decreasing TREM-2 levels resulted in defective

Table 1. Phenotype of TREM-2/DAP12 deficiencies in human and mouse

		Phenotype	
		In vitro	*In vivo*
Human	DAP12−/−	Defect of osteoclast formation	Bone cysts
	TREM-2−/−	Defect of osteoclast formation	Bone cysts
Mouse	DAP12−/−	Defect of osteoclast formation	Mild osteopetrosis, no bone cysts
	TREM-2−/−	Accelerated osteoclastogenesis	No osteopetrosis or bone cysts

osteoclastogenesis in-vitro. Together, these results suggest that TREM-2 is required for successful osteoclastogenesis. Finally, in vivo studies of DAP12−/− mice revealed the development of a mild increase in bone mass, consistent with a defect in osteoclast function in-vivo (Table 1) (Faccio, Zou, Colaianni, et al. 2003; Humphrey, Ogasawara, Yao, et al. 2004; Kaifu, Nakahara, Inui, et al. 2003).

All these studies demonstrate a role of TREM-2/DAP12 in osteoclastogenesis and reveal that a deficit of TREM-2/DAP12 may lead to a mild osteopetrosis. However, it is noteworthy that unlike the DAP12−/− mice, NHD patients have normal numbers of osteoclasts and do not have an obvious increase of bone mass but instead develop bone cysts (Table 1). Thus, it is possible that TREM-2/DAP12 have additional functions on bone modeling that have yet not been recognized in vitro and/or in vivo.

6. THE PARADOXICAL INHIBITORY FUNCTION OF TREM-2

Although the DAP12 signaling pathway is generally considered to trigger cell activation, recent data demonstrate that in some context DAP12-signaling downstream of TREM-2 may have an inhibitory effect on cell activation. Initial studies by Hamerman et al. demonstrated that DAP12−/− macrophages have an exaggerated response to TLR ligation, as if DAP12 inhibits macrophage responses to microbial components (Hamerman, Tchao, Lowell, et al. 2005). Since DAP12 associates with multiple receptors, it was unclear which DAP12-associated receptor mediates such inhibition. Recently, Hamerman et al. demonstrated that siRNA attenuation of TREM-2 expression in macrophages results in increased TLR responses (Hamerman, Jarjoura, Humphrey, et al. 2006). The generation of TREM-2-deficient mice has allowed the conclusive validation of the inhibitory function of TREM-2 on macrophages. Bone marrow derived macrophages from TREM-2−/− mice produce higher levels of the inflammatory cytokines TNF-alpha and IL-6 in response to the TLR agonists LPS, zymosan and CpG, demonstrating that TREM-2 inhibits the response to macrophages to TLR ligation (Turnbull, Gilfillan, Cella, et al. 2006). Moreover, parallel comparison of TREM-2−/− and DAP12−/− macrophages showed that TREM-2 deficiency largely accounted for the increased cytokine production observed in DAP12−/− mice, demonstrating that it is TREM-2 that is the operative receptor in the phenotype reported for DAP12−/− mice (Turnbull, Gilfillan, Cella, et al. 2006). The mechanisms by which DAP12 and TREM-2 inhibit cellular activation are unclear, although several interesting hypothesis have been proposed recently (Hamerman and Lanier 2006). In one model, the type of signal delivered by TREM-2/DAP12 depends on the avidity/affinity of TREM-2 ligands. High avidity/affinity ligands may induce extensive TREM-2/DAP12 clustering, leading to full activation of the DAP12 signaling cascade. In contrast, low avidity/affinity ligands may induce an abortive DAP12 pathway, which may cause recruitment without activation of downstream signaling mediators, effectively resulting in their sequestration. Alternatively, this abortive pathway may lead to recruitment of protein tyrosine phosphatases that actively inhibit cell activation (Figure 2). Although this model has not been demonstrated for TREM-2 or any other DAP12 associated receptors, it has been previously established for other activating receptors such as the Fc receptor alpha and the T cell receptor (Pasquier, Launay, Kanamaru, et al. 2005; Stefanova, Hemmer, Vergelli, et al. 2003).

Consistent with an inhibitory function of TREM-2, preliminary data from TREM-2−/− mice suggests that in mice, TREM-2 may inhibit osteoclastogenesis. When osteoclasts were derived in-vitro from TREM-2−/− mice, we observed accelerated osteoclastogenesis with more rapid fusion of the cells to form functional osteoclasts capable of resorbing bone ex-vivo (Table 1)

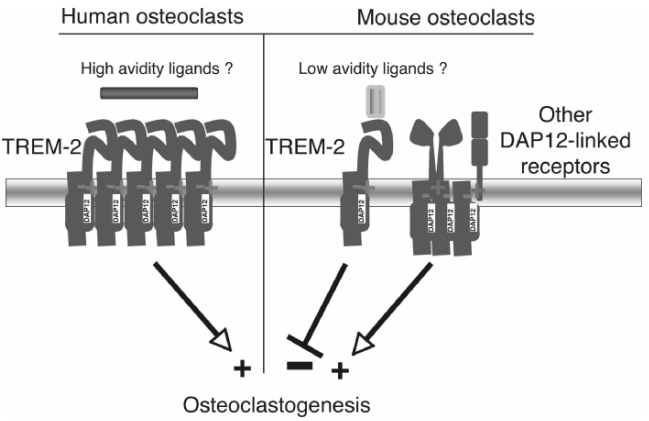

Figure 2. A model for the opposing functions of TREM-2/DAP12 in osteoclastogenesis. In human, TREM-2 is engaged by a high avidity/affinity ligand that induces extensive TREM-2/DAP12 clustering, leading to an activating signaling cascade. In mouse, TREM-2 is engaged by a low avidity/affinity ligand that induces abortive DAP12 signaling, leading to inhibition of osteoclastogenesis. In vivo, the activating signals mediated other DAP12 associated receptors overcome the inhibitory function of TREM-2. Thus, while TREM-2 deficiency accelerates osteoclastogenesis, DAP12 deficiency impairs it (*See Color Plate*).

(Colonna, unpublished results). Additionally, these mice do not have the osteopetrosis observed in DAP12−/− mice (Table 1). These data conflict directly with osteoclastogenesis data from the TREM-2- and DAP12-deficient NHD patients, and also the DAP12−/− mice. Based on the significant defect in osteoclastogenesis observed both in-vitro and in-vivo in DAP12-deficient mice, it is likely that there are other DAP12-associated receptors (in addition to TREM-2) that are operative in murine osteoclastogenesis (Figure 2). Moreover, it appears that while human TREM-2 activates osteoclastogenesis, mouse TREM-2 has an inhibitory role in the formation of osteoclasts. It is possible that, at least in our culture conditions, human TREM-2 is engaged by high avidity/affinity ligands that trigger activation, while mouse TREM-2 binds low affinity ligands that induce inhibition (Figure 2).

Thus, the role of TREM-2 in osteoclastogenesis remains unresolved, with human studies indicating that TREM-2 is required for the normal differentiation and function of osteoclasts, and mouse studies giving conflicting results as to the relationship between TREM-2, DAP12 and osteoclastogenesis. No doubt, understanding these opposing functions of TREM-2 will depend on the identification of TREM-2 ligands and their expression in bone.

7. TREM-2 LIGAND(S)

The ligand for TREM-2 is yet unknown, but several candidates have been proposed. A study by Daws et al. postulated a role for TREM-2 as a pattern recognition receptor specific for polyanionic microbial products. Soluble TREM-2 bound to bacteria, and bacteria bound to TREM-2 expressing cells. Additionally, these authors identified a putative endogenous ligand expressed by immortalized astrocytoma cell lines (Daws, Sullam, Niemi, et al. 2003). However, the specific molecules mediating these interactions have yet to be identified, and this will be critical to the further studies of TREM-2.

Studies by Hamerman et al. demonstrated that a putative TREM-2 ligand is expressed on bone-marrow derived macrophages (Hamerman, Jarjoura, Humphrey, et al. 2006). These studies extended from the observation that siRNA abrogation of TREM-2 expression by bone-marrow derived macrophages augmented inflammatory cytokine production, suggesting that bone marrow-derived macrophages express a TREM-2 ligand that engages TREM-2 and triggers an inhibitory signal. Hamerman et al. found that soluble TREM-2 receptor bound to bone-marrow derived macrophages and that this binding could be specifically blocked with a TREM-2 antibody. These data are promising; however, further studies will be required to identify the specific binding partner of TREM-2 expressed by macrophages.

Recent studies by Takegahara et al. suggest that TREM-2 ligand may be part of a multimeric complex. These author generated mice genetically deficient in PlexinA1, a molecule originally characterized for its role in axon guidance and cardiac morphogenesis (Takegahara, Takamatsu, Toyofuku, et al. 2006). They found that PlexinA1-deficient mice have a significant increase in bone mass as compared to wild type mice, and that this effect was likely mediated by interactions between PlexinA1 and a previously identified ligand, Semaphorin6D (Sema6D). Because Plexins often function in concert with a co-receptor, Takegahara et al. screened for interactions between the Plexin/Semaphorin system and potential candidate receptors that had previously been shown to act in bone homeostasis. This approach led to the identification of a receptor complex containing TREM-2, PlexinA1 and DAP12. Additional studies demonstrated that activation of PlexinA1 by Sema6D could be blocked by decreasing TREM-2 expression with siRNA. These data suggest that TREM-2 may function as part of a multimeric receptor complex, in which TREM-2 may bind Sema6D or, more likely, a second ligand associated with Sema6D. These results suggest that recognition of a distinct ligand by TREM-2 may require the presence of both a co-receptor such as PlexinA1 and its ligand, Sema6D.

8. CONCLUSION

The role of the TREM-2/DAP12 complex in normal bone modeling and osteoclast function is evidenced by a) the observation that genetic defects of human TREM-2 and DAP12 result in bone cysts b) the expression of TREM-2/DAP12 in pre-osteoclasts; c) the inability of human TREM-2- and DAP12-deficient pre-osteoclasts to develop into mature bone resorptive osteoclasts. In mouse, DAP12-deficiency impairs osteoclastogenesis and results in a mild osteopetrosis but no bone cysts were observed, suggesting that osteoclastogenesis may not be the only function controlled by TREM-2/DAP12, at least in human. Surprisingly, TREM-2-deficiency in mouse led to accelerated osteoclastogenesis in vitro without osteopetrosis or bone cysts in vivo, revealing an unexpected inhibitory function of mouse TREM-2. It is possible that in murine bone marrow-derived cultures TREM-2 is engaged by low avidity/affinity ligands that elicit an abortive DAP12 signaling. A complete understanding of the discrepancies between TREM-2 functions in human and mouse has been hampered by the failure to determine the specific ligands for the TREM-2, but recent data describing a multi-subunit receptor complex that includes Plexin A1, TREM-2 and Sema6D provide exciting insights into potential TREM-2 ligands. Ligand identification and the study of TREM-2-deficient mice will reveal the mechanisms involved in TREM-2-mediated bone modeling.

9. ACKNOWLEDGMENTS

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CHAPTER 14
ROLE OF CELL-MATRIX INTERACTIONS IN OSTEOCLAST DIFFERENTIATION

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1. ABSTRACT

Osteoclast and their mononuclear cell precursors are present within the bone microenvironment at sites of physiologic and pathologic bone resorption. Analysis of tissues from sites of bone resorption reveal that cells expressing the full morphological and functional properties of mature osteoclasts are restricted to the immediate bone surface. We hypothesize that in addition to cytokines, components of the bone matrix and specific cell surface receptors on osteoclasts and their precursors play an essential role in determining the genetic profile and functional properties of fully differentiated resorbing osteoclasts. We have employed expression profiling, with an *in vitro* model of matrix-dependent osteoclast differentiation, to identify the molecular pathways by which bone matrix-interactions induce terminal osteoclast differentiation and activation. In preliminary studies, we have identified unique genes and transcriptional pathways that are induced by interaction of osteoclast precursors with specific components of the mineralized bone matrix. The authenticity of the gene profiles, as markers of osteoclast differentiation and activation, have been provisionally validated using an *in vivo* animal bone implantation model and by examination of tissues from patients with specific forms of pathologic osteoclast-mediated bone resorption. The ultimate goal of our studies is to identify new molecular targets for inhibiting osteoclast-mediated bone loss in disorders of pathologic bone loss.

The early work of Walker et al. (Walker 1972) in parabiotic animals, and the subsequent studies of Burger et al. (Burger, Van der Meer, van de Gevel, et al. 1982) using a co-culture model with fetal bone rudiments and bone marrow-derived cells, have helped to establish that osteoclasts are derived from macrophage precursors of colony forming unit-macrophage (CFU-M lineage). As such, they share a common hematopoietic origin with other CFU-M lineage cells, including tissue macrophages that populate the lung (alveolar macrophages), liver (Kupfer cells), synovium (synovial macrophages) and other organs. They also share a common lineage

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with dendritic cells and macrophage polykaryons (foreign body giant cells) that are involved in immunoregulation and inflammation.

Much has been learned in recent years regarding the regulatory pathways and factors that control the commitment and differentiation of the CFU-M precursor for these phenotypically distinct cell types. With respect to the osteoclast, observations made in a variety of animal models and human disorders associated with defective osteoclast differentiation, have helped to dissect the regulatory mechanisms and commitment points in the differential pathways of macrophage and osteoclast differentiation (Roodman 1999; Suda, Nakamura, Jimi, et al. 1997; Teitelbaum 2000; Teitelbaum and Ross 2003). Under the influence of osteoclast-inducing hormones or cytokines, hematopoietic precursors present within the bone marrow commit to the monocyte/macrophage lineage and undergo proliferation (Roodman 1999; Suda, Nakamura, Jimi, et al. 1997; Teitelbaum 2000; Teitelbaum and Ross 2003). The hematopoietic growth factor, colony-stimulating factor-1 (CSF-1), acting via its receptor CSF-1R, plays an essential role in these initial events. At this early stage, the precursors retain their pluri-potential capacity to differentiate down the macrophage or osteoclast pathway. There is increasing evidence that local factors, including the cytokine milieu and the effects of cell-cell and cell-matrix interactions, determine the ultimate cytodifferentiation pathway of these cells and their commitment to osteoclast or macrophage differentiation.

Following commitment to monocyte/macrophage lineage, osteoclast precursors are recruited to the trabecular bone surface where they interact with bone lining cells that are of osteoblast lineage via adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) or vascular cell adhesion molecule-1 (VCAM-1) (Athanasou and Quinn 1990; Ishikawa, Hirata, Nishibayashi, et al. 1994; Veale, Rogers, and Fitzgerald 1995; Youssef, Triantafillou, Parker, et al. 1997). This leads to retraction of the lining cells and exposure of the bone surface (Athanasou and Quinn 1990; Ishikawa, Hirata, Nishibayashi, et al. 1994). Blockade of osteoblast-derived metalloproteinase (MMP) activity inhibits bone resorption and it has been suggested that the proteinase activity may be required for lining cell retraction and for proteolytic cleavage of type I collagen that reveals cryptic binding sites for $\alpha_v\beta_3$ integrin that are necessary for optimal preostoclast attachment and osteoclast activation (Karsdal, Fjording, Foged, et al. 2001; Messent, Tuckwell, Knauper, et al. 1998). Further evidence supporting a role for metalloproteinases in preostoclast attachment to the bone surface is provided by the studies of Zhao et al. (Zhao, Byrne, Boyce, et al. 1999). These studies showed that osteoclast-mediated bone resorption induced by parathyroid hormone (PTH) was markedly impaired in MMP-13-resistant mutant mice. Similar results have been observed by Holliday et al. (Holliday, Welgus, Fliszar, et al. 1997) who used an *in vitro* model to show that collagenase inhibition decreases osteoclast-mediated bone resorption.

The active process of osteoclast-mediated bone resorption is initiated by attachment of the osteoclast to the bone matrix, followed by cytoskeletal reorganization and cell surface membrane polarization and specialization. The attachment of the osteoclast involves interaction of cell surface integrins, including the vitronectin receptor $\alpha_v\beta_3$, the collagen/laminin receptor $\alpha_2\beta_1$ and the vitronectin/fibronectin receptor $\alpha_v\beta_1$, with bone matrix components (Duong, Lakkakorpi, Nakamura, et al. 2000; Duong and Rodan 1999; Helfrich, Nesbitt, Lakkakorpi, et al. 1996). The osteoclast expresses the β1-, β3- and β5-containing integrins. During osteoclast differentiation, the β5 and β3 are reciprocally regulated, with down-regulation of β5 and up-regulation of the β3 integrin (Inoue, Namba, Chappel, et al. 1998; Inoue, Ross, Erdmann, et al. 2000).

The $\alpha_v\beta_3$ vitronectin receptor mediates adhesion to a wide variety of bone matrix components, including osteopontin, bone sialoproteins and denatured collagen, via interaction with RGD peptides (Duong, Lakkakorpi, Nakamura, et al. 2000; Duong and Rodan 1999; Flores,

Heinegard, Reinholt, et al. 1996; Helfrich, Nesbitt, Lakkakorpi, et al. 1996). Despite the high levels of expression of the $\alpha_v\beta_3$ integrin in mature osteoclasts, this binding protein is not essential for pre-osteoclast attachment, as evidenced by the findings in the β3 knockout mouse, which express increased numbers of osteoclasts on the bone surfaces (McHugh, Hodivala-Dilke, Zheng, et al. 2000). The failure of the β3 knockout mice to adequately resorb bone implies that signaling through this protein is essential for full/optimal functional activation of the osteoclast. Interestingly, the $\beta5^{-/-}$ mice exhibit increased osteoclast maturation and activity after ovariectomy indicating that this integrin, similar to the β3 integrin, is not required for pre-osteoclast attachment. The capacity of the β1-, β3- and β5-containing integrins to compensate for each other in various β integrin knock-out mice may account for these paradoxical observations.

After attachment of the osteoclast to the bone surface, the cells undergo cytoskeletal reorganization with formation of highly specialized membrane structures that are essential for the acquisition of resorbing activity. These changes consist of formation of the so-called ruffled border and clear zone of attachment (Athanasou 1996; Roodman 1996; Teitelbaum 2000). The interface between the ruffled border and adjacent bone surface forms an acidic environment that contributes to the dissolution of the bone mineral phase. Protons for generating this acidic environment are provided by carbonic anhydrase II (CarII) and a pH gradient is maintained by a proton ATPase and chloride channel, which are present in the ruffled border membrane (Kornak, Kasper, Bosl, et al. 2001; Taranta, Migliaccio, Recchia, et al. 2003; Vaananen, Karhukorpi, Sundquist, et al. 1990). Cathepsin-k, MMP-9, and tartrate resistant acid phosphatase (TRAP) are among the enzymes that are expressed in these cells and contribute to the resorption of the extracellular matrix component of bone (Teitelbaum 2000; Tezuka, Nemoto, Tezuka, et al. 1994). However, these activities are not unique to the osteoclast. For example, under certain conditions, TRAP activity is expressed in a variety of cell types, including tissue macrophages and macrophage polykaryons, that are not involved directly in bone resorption (Athanasou 1996; Chambers 2000; Hattersley and Chambers 1989). In our own studies, we have used *in situ* hybridization and immunostaining techniques to characterize the differential phenotype of macrophages, macrophage polykaryons and osteoclasts in tissue samples from patients with rheumatoid arthritis (RA) and peri-implant osteolysis after total joint replacement (Shen, Crotti, McHugh, et al. 2006). These analyses confirm that many osteoclast-associated genes are expressed in mono- and multinucleated cells with phenotypic features of macrophages or macrophage polykaryons. In contrast, genes, including the β3 integrin and calcitonin receptor, are specifically up-regulated in mono- and multinucleated osteoclast-like cells attached to the bone surface. These findings, as well as the work of others have led us to hypothesize that in addition to cytokines, components of the bone matrix and specific cell surface receptors on osteoclasts and their precursors play an essential role in determining the genetic profile and functional properties of fully differentiated resorbing osteoclasts.

We have employed expression profiling with an *in vitro* model of matrix-dependent osteoclast differentiation to identify the molecular pathways by which bone matrix-interaction induce terminal osteoclast differentiation and activation. Purified mouse monocyte macrophages were isolated from bone marrow of 5 week old, male C57/B6 mice. Osteoclast differentiation was induced by treatment with CSF-1 and receptor activator of NF-κB (RANKL) (R&D Systems) in cells grown on tissue culture plastic dishes, hydroxyapatite coated surfaces (Osteologic, BD Bioscience) or in wells containing discs of calvarial bone, as previously described (McHugh, Shen, Fleming, et al. 2005; McHugh, Crotti, Shen, et al. 2006; Shen, Fajardo, Tsay, et al. 2004). After 5 days, adherent cells were fixed with 4% paraformaldehyde for histological examination or RNA was purified for quantitative RT-PCR and genomic profiling using oligonucleotide array expression profiling on Affymetrix Mouse Genome

430 2.0 GeneChips. Following microarray normalization and gene comparison, hierarchical clustering was performed and pathways regulated by bone matrix adherence identified using the Ingenuity Pathway Analysis program. In preliminary studies, we have identified unique genes and transcriptional pathways that are induced by interaction of osteoclast precursors with specific components of the mineralized bone matrix. The authenticity of the gene profiles as markers of osteoclast differentiation and activation has been provisionally validated using an *in vivo* animal bone implantation model and by examination of tissues from patients with specific forms of pathologic osteoclast-mediated bone resorption. The ultimate goal of these studies is to identify new molecular targets for inhibiting osteoclast-mediated bone loss in disorders of pathologic bone loss.

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CHAPTER 15
POSITIVE AND NEGATIVE ROLES OF IL-6, STAT3, AND SOCS3 IN INFLAMMATORY ARTHRITIS

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1. INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of multiple joints, resulting in mononuclear cell infiltration, development of pannus, progressive cartilage destruction and bone erosion. Although the cause of RA remains unknown, it has been suggested that cytokines, especially proinflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin (IL)-1, and IL-6 derived from activated synovial cells, play a pivotal role in the pathology of the disease (Feldmann, Brennan, and Maini 1996a,b). Among these cytokines, TNF-α has been most extensively investigated as a target in the efforts to treat RA. Anti-TNF-α mAbs markedly ameliorate joint involvement in the majority of patients with RA (Elliott, Maini, Feldmann, et al. 1994a,b). It is well known that IL-6 is one of a major target gene of TNF-α. IL-6 has been proposed to contribute to the development of arthritis; IL-6 induces proliferation of synovial fibroblastic cells (Mihara, Moriya, Kishimoto, et al. 1995) and formation of osteoclasts, in association with soluble IL-6 receptors (Tamura, Udagawa, Takahashi, et al. 1993). Involvement of IL-6 in RA is also suggested by recent reports of IL-6-gene-disrupted mice that were resistant to antigen-induced arthritis (AIA) (Boe, Baiocchi, Carbonatto, et al. 1999; Ohshima, Saeki, Mima, et al. 1998) and collagen-induced arthritis (CIA) (Alonzi, Fattori, Lazzaro, et al. 1998). Therefore, IL-6 could be another therapeutic target of RA. Recently, anti-IL6 receptor antibodies have been successfully trialed as a therapy for RA patients (Yoshizaki, Nishimoto, Mihara, et al. 1998). However, the mechanism of suppression of RA by anti-IL-6 therapy has not been fully understood. We investigated the role of IL-6 and its signal regulator, SOCS3 on inflammatory arthritis and osteoclastgenesis using mouse models.

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2. IL-6 AND ITS SIGNAL TRANSDUCTION

IL-6 and its related cytokines, including leukemia inhibitory factor (LIF), oncostatin M (OSM), and IL-11, preferentially activate JAK tyrosine kinases and the STAT3 transcription factor (Figure 1). STAT3 activation was found exclusively in synovial tissue from RA, but not osteoarthritis (OA), patients (T. Shouda, T. Yoshida, T. Hanada, et al. 2001). A mouse line of mutated gp130, in which STAT3 was hyperactivated, developed an RA-like joint disease with increased production of Th1-type cytokines and Igs of the IgG2a and IgG2b classes (Atsumi, et al. 2002). Therefore, elucidation of the regulatory mechanisms of the JAK-STAT3 pathway is important in understanding the inflammation associated with RA.

Several target genes of STAT3 have been identified; proteins that are involved in cell survival and proliferation such as Bcl-2, Bcl-xL, mcl-1, Fas, cyclin D1, cyclin E1, and p21. In addition, other transcription factors, including c-myc, c-jun, and c-fos, are themselves STAT3 targets (Hirano, Ishihara, Hibi 2000). VEGF and TGFβ have been shown to be a target of STAT3 and to contribute to angiogenesis and fibrosis, respectively (Xu, Briggs, Park, et al. 2005; Ogata, Chinen, Yoshida, et al. 2006). Another important target of STAT3 is the tissue inhibitor of metalloproteinases-1 (TIMP-1), which inhibits MMPs and could be involved in tissue remodeling. Importantly, RANKL, which is an essential factor for osteoclastgenesis, is also shown to be a target of STAT3 (O'Brien, et al. 1999). Thus, hyperactivation of STAT3 in synovial fibroblasts induces both hyperplasia of synovial tissues and osteoclast accumulation.

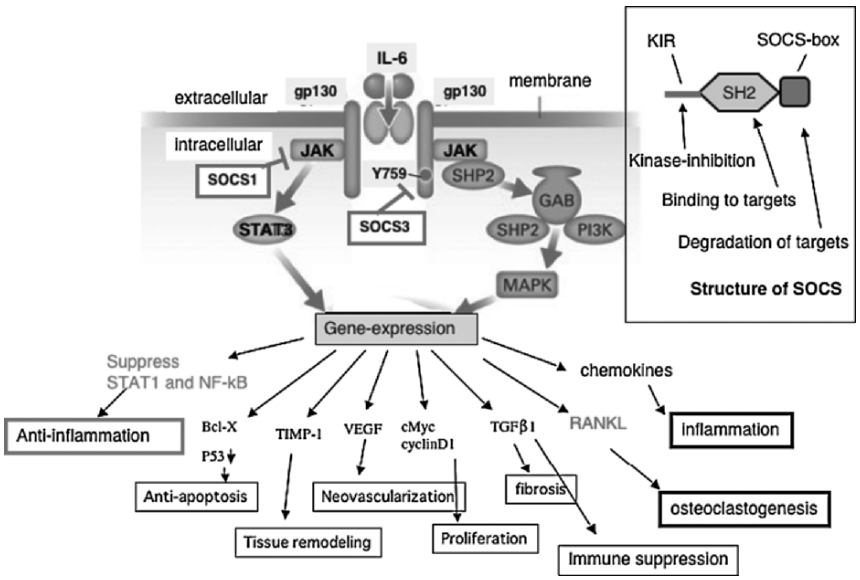

Figure 1. Signal transduction pathway and target genes of IL-6. Inset shows the structure of SOCS.

3. SOCS3; A NEGATIVE REGULATOR OF IL-6 AND STAT3

We recently cloned SOCS1 and SOCS3 which inhibit JAK tyrosine kinase activity (Kubo, Hanada, and Yoshimura 2003). Many reports have indicated that SOCS3 is induced not only by various inflammatory and anti-inflammatory cytokines such as IL-6, IFNγ, IL-10 but also by lipopolysaccharide (LPS), and that SOCS3 negatively regulates STAT functions (Yoshimura, Nishinakamura, Matsumura, et al. 2005).

Both SOCS1 and SOCS3 can inhibit JAK tyrosine kinase activity since they have the kinase inhibitory region (KIR) in their N-terminal domain, which is proposed to function as a pseudosubstrate (Yoshimura, Nishinakamura, Matsumura, et al. 2005) (Figure 1). While SOCS1 directly binds to the activation loop of JAKs through its SH2 domain, the SOCS3 SH2 domain binds to the cytokine receptor. The SOCS3 SH2 domain has been shown to bind to Y757 of gp130. SOCS3 also suppresses LIF, G-CSF, and leptin signalining, all of which activate STAT3. Thus, SOCS3 is a relatively specific inhibitor to STAT3.

If STAT3 is a promoting factor of inflammatory arthritis, SOCS3 could be a negative modulator of arthritis. We attempted to suppress murine RA models using adenovirus carrying SOCS3 or dnSTAT3. We found that both SOCS3 and dnSTAT3 overexpression suppress mononuclear cell infiltration and pannus formation, as well as bone destruction induced by antigen-induced or collagen-induced arthritis (Figure 2a). Local expression of SOCS3 and dnSTAT3 suppressed STAT3 activation

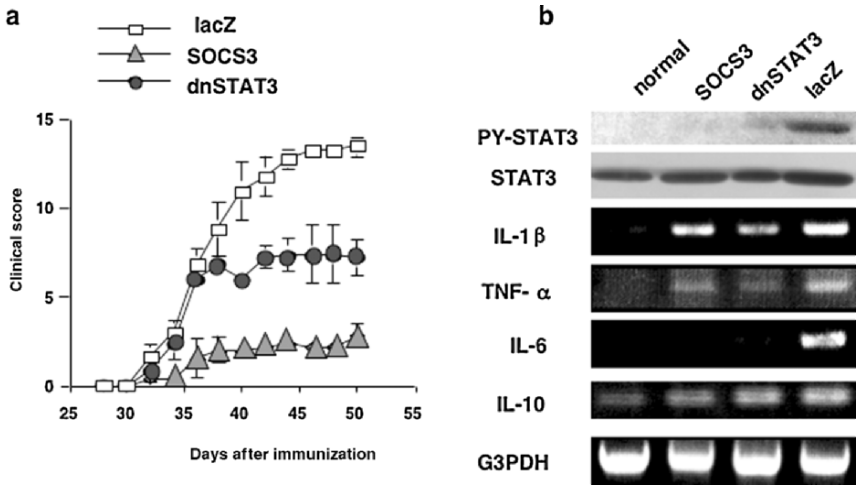

Figure 2. Therapeutic effects of SOCS3-virus injection in murine arthritis models. (A) DBA/1J mice were immunized by intradermal injection at the base of the tail with 100μg of bovine type II collagen with Freund's complete adjuvant (day 0). On day 21, mice received a booster dose of intradermal injection. On day 28, LPS was injected intraperitoneally and adenoviruses were injected into ankle joints and wrists. Severity of disease was assessed every other day using an established macroscopic scoring system ranging from 0 to 4 for each leg. Data represent mean clinical score of 5 mice from each group. (B) Effect of SOCS3 and dnSTAT3 on STAT3 activation and cytokine production in the antigen-induced arthritis model. SOCS3-, dnSTAT3- or lacZ-viruses were infected on day 6 after the initial mBSA immunization. Ankle joints were removed and homogenized 28 days after immunization. The ankle joint of an untreated mouse was used as a normal control (normal). Cell extracts were immunoblotted with anti-phosphorylated STAT3 or anti-STAT3 antibodies. Twenty-eight days after the first immunization, total RNA was extracted from ankle joints of mice infected with adenoviruses at day 6. The ankle joint of an untreated mouse was used as a normal control.

and RT-PCR analysis confirmed strong suppression of IL-6 production by SOCS3 and dnSTAT3 overexpression (Figure 2b). However, TNFα, IL-1β, and IL-10 levels were not affected in arthritic joints.

4. ROLE OF IL-6, STAT3 AND SOCS3 IN SYNOVIAL FIBROBLASTS

To elucidate the significance of STAT3 activation and SOCS3 induction in RA, we overexpressed dnSTAT3 and SOCS3 in RA synoviocytes isolated from patients, using adenoviral gene transfer (Shouda, Yoshida, Hanada, et al. 2001). We infected cultured synovial fibroblasts *in vitro* with adenovirus carrying SOCS3 (AxCASOCS3), dominant-negative (dn)STAT3 (AxCAdnSTAT3), or lacZ (AxCAlacZ). Adenovirus was inoculated at multiplicity of infection (MOI) = 50, and 100% of cells were infected as assessed by lacZ staining (data not shown). AxCASOCS3 and AxCAdnSTAT3, but not AxCAlacZ infection strongly suppressed proliferation of synovial fibroblasts in 10% FCS (Figure 3a). Thus, proliferation of synovial fibroblasts is strictly dependent on JAK/STAT3 pathway. Suppression of proliferation by SOCS3 and dnSTAT3 was not observed in NIH-3T3 fibroblasts (data not shown), suggesting that STAT3-dependent growth is a specific feature of synovial fibroblasts.

We next investigated the effect of SOCS3 and dnSTAT3 on secretion of proinflammatory cytokines. The levels of TNF-α, IL-1β, and IL-6 were analyzed in the conditioned medium (CM) of AxCASOCS3-, AxCAdnSTAT3- and AxCAlacZ-infected RA synovial fibroblasts (Figure 3c). Serum- as well as TNFα-induced IL-6 production were significantly decreased by infection with AxCASOCS3 and AxCAdnSTAT3, but not with AxCAlacZ (controls). However,

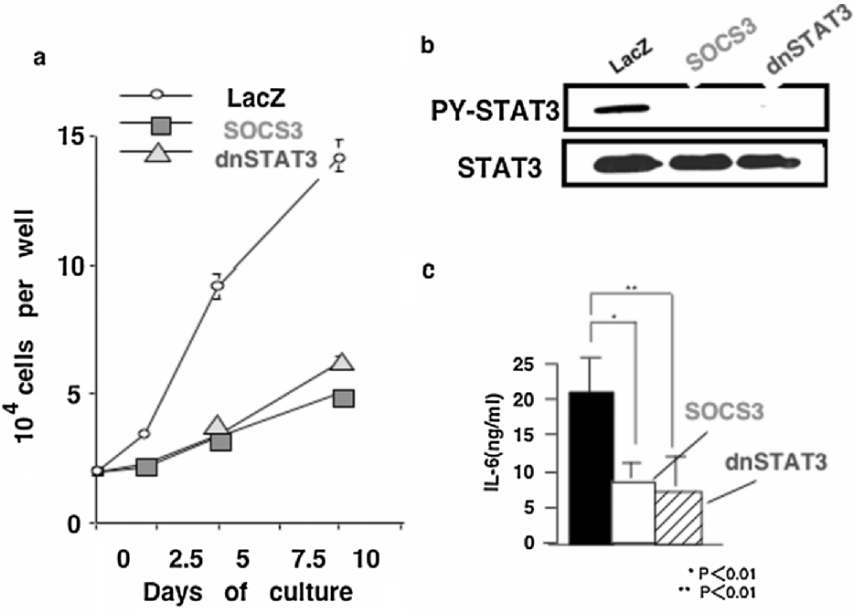

Figure 3. Effects of dnSTAT3 and SOCS3 overexpression on proliferation and cytokine secretion of RA synovial fibroblasts. (A) Cell growth curves of rheumatoid synoviocytes inoculated with LacZ-, dnSTAT3- or SOCS3-adenovirus (MOI = 50). (B) STAT3 phosphorylation was detected by immunoblotting with anti-psohporylated STAT3 antibody. (c) IL-6 concentrations in conditioned medium were detected by ELISA. Data are expressed as mean ± SD derived from three separate experiments (*P < 0.01 *vs*. LacZ).

TNF-α and IL-1β levels were not altered by infection with AxCASOCS3 and AxCAdnSTAT3 (Figure 3c). These data suggest that JAK/STAT pathway is involved in IL-6 production in RA synovial fibroblasts, and that SOCS3- and dnSTAT3-adenovirus can suppress not only IL-6 signaling but also a part of TNFα signaling.

5. IL-6, SOCS3 AND MACROPHAGE ACTIVATION

IL-6 is a pro-inflammatory cytokine that plays a progressive role in many inflammatory diseases, while IL-10 is an immunoregulatory cytokine that has potent anti-inflammatory activity. Although the transcription factor STAT3 is essential for the function of both IL-6 and IL-10 (Takeda, et al. 1999), it is not clear how these two cytokines exhibit such opposite functions. Recently, we demonstrated that, at least in macrophages, SOCS3 is a key regulator of the divergent action of these two cytokines. In macrophages lacking the SOCS3 gene, or carrying a mutation of the SOCS3 binding site (Y759F) in gp130, not only IL-10, but also IL-6, suppressed LPS-induced TNFα production (Yasukawa, et al. 2003) (Figure 4). SOCS3 protein was strongly induced by both IL-6 and IL-10 in the presence of LPS, but selectively inhibited IL-6 signaling, binding to, R/gp130 but not to IL-10R (Yasukawa, et al. 2003). These data indicate that SOCS3 selectively blocks IL-6 signaling, interfering with its ability to inhibit LPS signaling. Consistent with this, mice specifically lacking the SOCS3 gene in macrophages and neutrophils are resistant to acute inflammation as modeled by LPS-shock. This phenotype is the complete opposite to that of macrophages in STAT3 conditional KO mice that are more sensitive to LPS stimulation and produce more TNFα in response to LPS (Takeda, et al. 1999).

Others have shown that IL-6 strongly activates STAT1 and induces the expression of IFN-responsive genes in SOCS-3 deficient macrophages, implying that IL-6 might mimic the action of IFNs (Lang, et al. 2003; Croker, et al. 2003). Interestingly, these reports also demonstrated that the absence of SOCS3 in macrophages changes the original function of IL-6. Therefore, all three studies indicate that SOCS3 is an important regulator to maintain a specific biological function on gp130-related cytokines in vivo. From such an interesting biochemical and biological function of SOCS3, we may be able to convert inflammatory cytokine IL-6 to an anti-inflammatory cytokine by suppressing the expression of SOCS3 in macrophages.

6. ROLE OF SOCS3 IN OSTEOCLASTOGENESIS

SOCS3 expression is also upregulated by RANKL in bone marrow-derived monocytes (BMMs) (Hayashi, Kaneda, Toyama, et al. 2002). To determine the physiological role of SOCS3 in osteoclastogenesis, we used a conditional knockout (KO) approach, since SOCS3-deficient mice die during embryonic development as a result of placental defects (Yoshimura, Nishinakamura, Y. Matsumura, et al. 2005). To generate a monocyte-specific deletion, a mouse line in which the Cre cDNA was knocked-in at the lysozyme M gene locus (LysM-Cre mice) (Clausen, Burkhardt, Reith, et al. 1999) was crossed with SOCS3fl/fl mice. SOCS3 mRNA was induced by RANKL treatment within 5 hr in BMMs from SOCS3-WT, although it could not be detected in those from SOCS3-KO mice (Ohishi, Matsumura, Aki, et al. 2005). Furthermore, we confirmed that SOCS3 protein was not detected in osteoclasts of SOCS3-KO mice (Ohishi, Matsumura, Aki, et al. 2005).

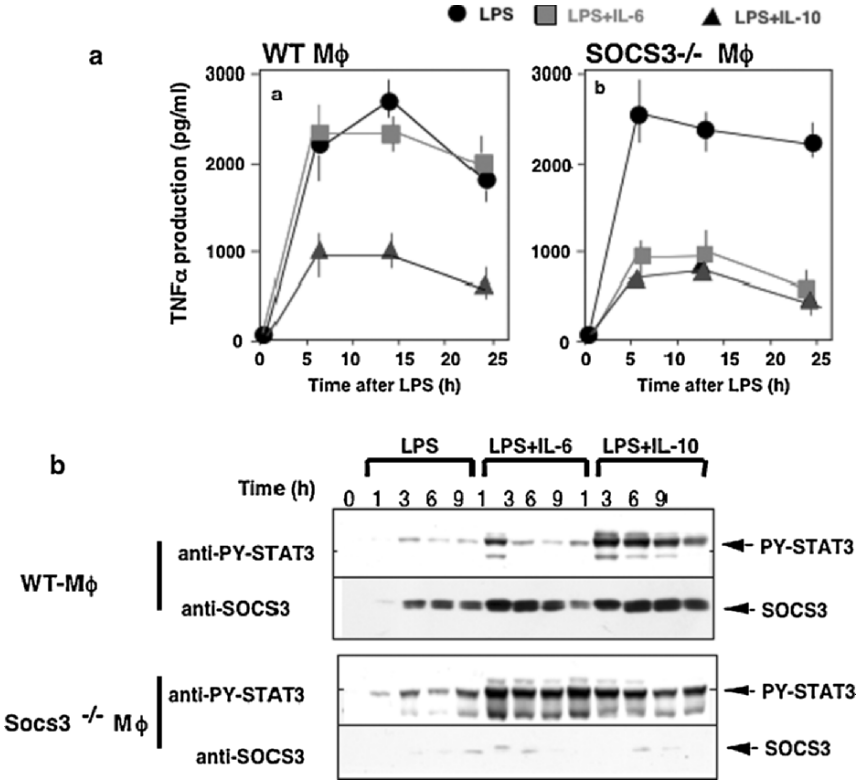

Figure 4. IL-6 suppresses LPS-induced TNFα production from macrophages from LysCre/flox/flox mice. (A) Mice were intraperitoneally injected with thioglycollate, and 3 days later peritoneal macrophages were isolated. Macrophages from wild type mice (WT-Mϕ) or LysCre/flox/flox mice (SOCS3−/−Mϕ) were stimulated with 100 ng/ml LPS in the presence of 100 ng/ml IL-6 or IL-10 for indicated periods. Concentrations of TNFα in the culture supernatants were measured by ELISA. (B) WT or SOCS3−/− macrophages were stimulated with 100 ng/ml IL-6 or IL-10 in the presence or absence of 100 ng/ml LPS for indicated periods. Total cell extracts were immunoblotted with anti-phosphorylated STAT3, anti-STAT3, or anti-SOCS3 antibodies.

This indicates that SOCS3 may play a role in osteoclast differentiation induced by RANKL, although the mechanism is unclear. We compared the inhibitory effect of IFNγ, IFNβ and IL-6 on osteoclast differentiation between wild-type and SOCS3-deficient BMMs. SOCS3 does not play a major regulatory role in IFNs sensitivity. IL-6 did not affect osteoclast differentiation of wild-type BMMs at a low concentration (0.1 ng/ml), but partially suppressed it at higher concentrations (≥1 ng/ml). In contrast, IL-6 strongly suppressed the osteoclast differentiation of SOCS3-deficient BMMs even at the low IL-6 concentrations and completely blocked it at higher concentrations (Figure 5a).

To define the molecular mechanism of this phenomenon, we examined the levels of TRAF6 and c-Fos, as well as the phosphorylation of IkB, STAT1, and STAT3 (Figure 5b). IL-6-induced phosphorylation of STAT3, as well as STAT1, was enhanced in SOCS3-KO BMMs. In wild-type BMMs, TRAF6, c-fos and IkB levels were not much affected by IL-6 pretreatment, whereas the expression level of TRAF6 and IkB, but not c-fos were

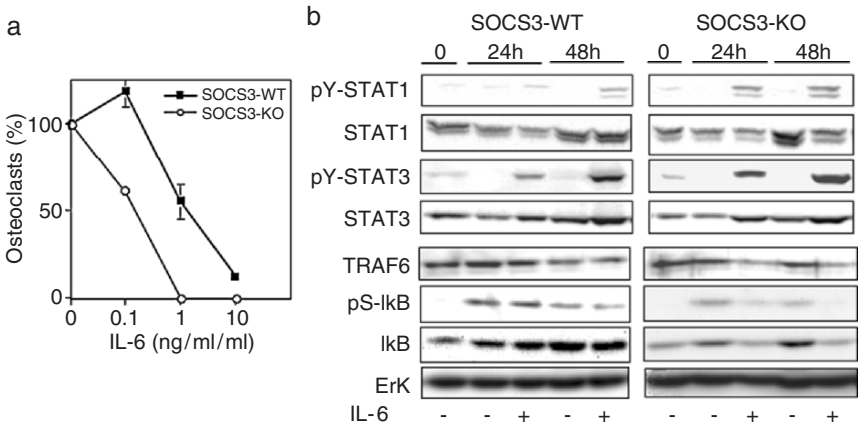

Figure 5. SOCS3-deficient BMMs are hyper-responsive to IL-6. (A) BMMs derived from both SOCS3-WT and SOCS3-KO mice were cultured for three days in the presence of RANKL (100 ng/ml) and M-CSF (100 ng/ml) and indicated concentrations of IL-6. Cells were fixed and stained with TRAP. The numbers of TRAP-positive MNCs were counted. The number of TRAP-positive MNCs in the cultures without IL-6 is valued as 100%. (b) Effects of IL-6 on STAT and TRAF6-NF-kB activation in SOCS3-KO BMMs. BMMs from SOCS3-WT and SOCS3-KO mice were cultured with M-CSF (100 ng/ml) and RANKL (100 ng/ml) with or without IL-6 (1ng/ml). Whole-cell extracts were immunoblotted with the indicated antibodies. (B) Suppression of TARF6 and IκB expression level and RANKL -induced IκB phosphorylation by IL-6 in SOCS3-deficient BMMs. Whole-cell extracts from BMMs were immunoblotted with the indicated antibodies.

significantly reduced in the presence of IL-6 in SOCS3-deficient BMMs (Figure 5b). Moreover phosphorylation of IkB was diminished by IL-6 pretreatment in SOCS3-deficient BMMs. Consisntent with these data, RANKL-induced nuclear translocation of RelA (p65), a subunit of NF-kB was inhibited by IL-6 in SOCS3-deficient BMMs. These data indicate that RANKL signaling was severely suppressed by IL-6 in SOCS3-deficient BMMs, resulting in the suppression of osteoclast differentiation.

Next we examined the role of SOCS3 in vivo using LysM-Cre:SOCS3fl/fl mice. Unlike osteoclastogenesis in vitro, bone density and mass were not significantly different between SOCS3-KO and control SOCS3-WT mice at normal conditions in vivo. In order to assess the role of SOCS3 in the regulation of inflammatory bone destruction, we used LPS-induced bone destruction model (Takayanagi, Ogasawara, Hida, et al. 2000). LPS-induced bone destruction was less severe, but not significantly in SOCS3-KO mice than in SOCS3-WT mice. However, when exogenous IL-6 was subcutaneously administered at the same site of LPS injection, bone destruction in SOCS3-KO mice was strongly suppressed, which was not observed in control SOCS3-WT littermates. These results are consistent with in vitro experiments that IL-6 strongly suppressed osteoclast differentiation of SOCS3-deficient BMMs.

7. ROLE OF SOCS3 AND STAT3 ON Th3 DIFFERENTIATION

RA is an autoimmune diseases, thus activated and regulatory T cells play important roles in initiation and promotion of RA. Thus we investigated the role of SOCS3 and STAT3 on T cell differentiation.

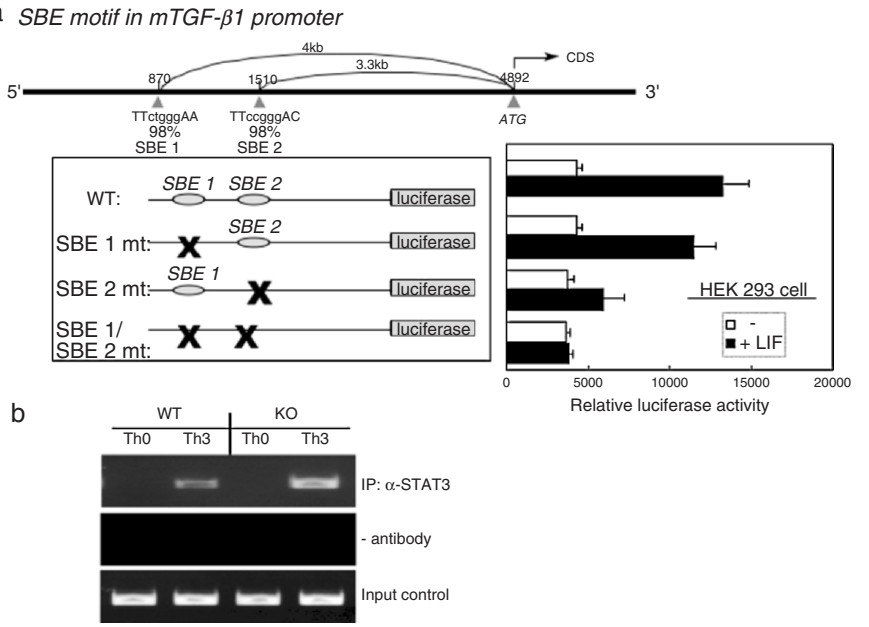

Figure 6. STAT3 enhances *TGF-β1* promoter activity. (A) Structure of TGFβ-1 promoter and STAT3-binding element (SEB), and the effects of point mutations introduced into the SBE-1 and SBE-2 elements. HEK293 cells were transiently transfected with WT or mutant promoter-reporter plasmids, and β-gal plasmid. Cells were stimulated with 10 ng/ml LIF and luciferase activities were measured. Luciferase activities were normalized by β-gal activities and expressed as fold induction to control cultures defined as 1.0. (B) ChIP assay to compare STAT3 recruitment to *TGF-β1* promoter (SBE-1 site) between T cells differentiated with Th0 (anti-CD3, ant-CD38, anti-IFNγ and anti-IL-12) and Th3 (anti-CD3, anti-CD28 antibodies plus TGF-β1, IL-4 and IL-10) conditions from WT and cKO mice. Anti-STAT3 antibody immunoprecipitates were used as templates for PCR cells. The final DNA extractions were amplified using pairs of primers that cover the STAT3 binding site (SBE-1) in the *TGF-β1* promoter region. G3PDH levels were determined by PCR using samples before immunoprecipitation as input control.

In the analysis of *Lck* promoter-driven SOCS3-transgenic mice, the high expression of SOCS3 in helper T cells led to skewing to Th2-type differentiation. This is probably because SOCS3 binds to IL-12Rβ2 and inhibits IL-12 mediated STAT4 activation, thereby blocking Th1 development (Seki, Inoue, Nagata, et al. 2003). Importantly, SOCS3 levels were high in T cells from allergic disease patients (Seki, Inoue, Nagata, et al. 2003). These observations implied that SOCS3 might be crucial for helper T cell differentiation and activation. To define the physiological roles of SOCS3 in T cells, we generated T cell-specific SOCS3 conditional knockout mice (Kinjyo, Inoue, Hamano, et al. 2006). Mice lacking SOCS3 in T cells showed reduced immune responses not only to OVA-induced airway hyperresponsiveness (Th2 diseases) but also to *Leishmania major* infection (Th1 disease). *In vitro* differentiation experiments revealed that SOCS3-deficient $CD4^+$ T cells differentiate into Th1 and Th2 cells normally, but produced more TGF-β1 and IL-10, but less IL-4 than control T cells, suggesting preferential Th3-like differentiation.

To address whether STAT3 is critical for IL-10 and TGF-β1 production in $CD4^+$ T cells, we introduced a constitutively activated STAT3 (STAT3c) or a dominant negative STAT3

(dNSTAT3) into $CD4^+$ T cells using a bicistronic retroviral vector. The retrovirus vectors were infected into nonpolarized $CD4^+$ T cells, which were stimulated with plate-bound anti-CD3ε mAb and anti-CD28 mAb, and on day 4, GFP-positive cells were sorted. The expression of myc-STAT3 in the sorted GFP-positive $CD4^+$ T cells was confirmed by Western blotting. These cells were cultured under Th0 or Th3-differentiating condition for 7 days and analyzed for cytokine production upon re-stimulation. We found introduction of STAT3c into $CD4^+$ T cells resulted in higher TGF-β1 and IL-10 production. In contrast, dNSTAT3-GFP infected $CD4^+$ T cells showed less production of TGF-β1 and IL-10. These data indicate that STAT3 activation is positively involved in the production of TGF-β1 and IL-10 in $CD4^+$ T cells.

We then investigated whether STAT3 could directly regulate the *TGF-β1* promoter activity. The 4.1kb fragment of the 5'-flanking region of the *murineTGF-β1* gene was fused to the luciferase expression vector, and promoter activity was examined in HEK293 cells by transient transfection. Luciferase gene expression was enhanced by LIF, suggesting that this 4.1kb 5'-fragment of the *TGF-β1* gene contained STAT3 responsive elements (Figure 6a). By searching for potential STAT3-binding sites with the consensus sequences, $TTC/A(N)_3G/TAA$, two candidates of STAT3-binding sites were identified in the 4.1kb *TGF-β1* 5'-flanking region. The two sites were at positions −3155 and −2515 upstream of the transcription initiation site in the *TGF-β1* promoter designated STAT3-binding element-1 (SBE-1) and SBE-2, respectively. To determine the significance of these elements, mutations were introduced into the SBE-1 and/or SBE-2 sites. A mutant promoter lacking both SBE-1 and SBE-2 did not respond to LIF stimulation anymore, while constructs containing single SBE site still responded to STAT3 (Figure 6a). These results indicate that the two SBE sites of *TGF-β1* promoter are important for STAT3 dependent activation.

To confirm STAT3 binding to the *TGF-β1* promoter in T cells, ChIP assay was performed (Figure 6b). Chromatin-DNA complex was immunoprecipitated with anti-STAT3 antibody, then, STAT3 binding to the *TGF-β1* promoter was analyzed using pairs of specific primers spanning the STAT3-binding sites. STAT3 was actually bound to the *TGF-β1* promoter region containing SBE-1 site in T cells. Then STAT3 recruitment to the *TGF-β1* promoter was compared between WT and SOCS3-deficient $CD4^+$ T cells using ChIP-assay. As shown in Figure 6b, STAT3 was recruited to the SBE regions, but not to a non-SEB region, in the *TGF-β1* promoter region under Th3, but not Th0, differentiating conditions. Importantly, about three times more STAT3 was recruited to the *TGF-β1* promoter in SOCS3-deficient $CD4^+$ T cells than in WT $CD4^+$ T cells under Th3 differentiating condition (Figure 6b). These data suggest that SOCS3 probably regulates the production of TGF-β1 through appropriate tuning of STAT3 activation in $CD4^+$ T cells.

8. ROLE OF SOCS3 ON Th17 DIFFERENTIATION

Recently, importance of another effector T cells, Th17 different from Th1 has been implicated in autoimmune diseases including RA. Th17 produced large amount of IL-17 and TNFα, but low levels of IFNγ. Th17 is induced by IL-23 or TGF-β plus IL-6. One of the important role of IL-6 on RA may be induction of Th17 in combination with TGF-β. To define the role of SOCS3 on Th17 induction, we used SOCS3-deficeint T cells. Our collaborators showed that SOCS3-deficiency in T cells resulted in stronger Th17 differentiation induced by both IL-23 and TGF-β+IL-6 (Chen, Laurence, Kanno, et al. 2006). Although the mechanism is still unclear, SOCS3 has been shown to be a negative regulator of Th17 induction.

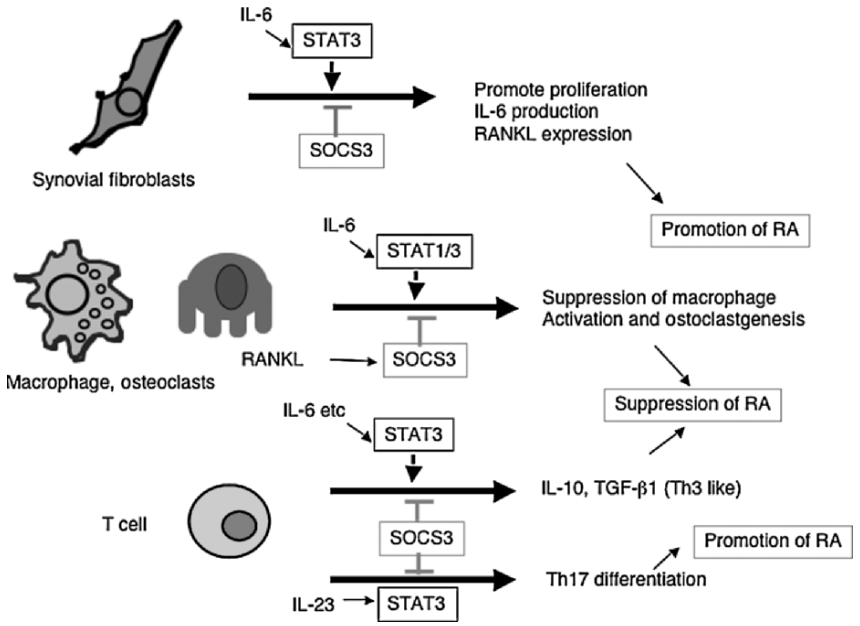

Figure 7. Summary of the role STAT3 and SOCS on RA development.

9. CONCLUSION

In summary, the IL-6-STAT3-SOCS3 system plays multiple roles in RA development (Figure 7). The IL-6-STAT3 pathway promotes RA by enhancing proliferation of synovial fibroblasts and RANKL expression. This pathway also promotes Th17 induction from naïve T cells. SOCS3 negatively regulates these processes. On the other hand, IL-6 inhibits oseoclastgenesis, but usually this inhibitory effect is not strong because SOCS3 protects osteoclasts from IL-6-STAT1/3 mediated suppression of RANKL signals. Furthermore, STAT3 enhances TGF-β1 production, while SOCS3 inhibits it. Thus STAT3/SOCS3 system regulates Th3-like Treg production. Our data suggest that STAT3 positively and SOCS3 negatively regulates production of both effector Th17 and suppressor Th3. Therefore, it is not clear at present whether suppression of IL-6/STAT3 in T cells is a benefit for suppression of RA. However, suppression or activation of STAT3/SOCS3 system in selected cells could be therapeutic for RA in the next generation.

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CHAPTER 16
CONTROL OF OSTEOCLAST ACTIVITY AND BONE LOSS BY IKK SUBUNITS: NEW TARGETS FOR THERAPY

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1. ABSTRACT

Transcription factor NF-κB has been well recognized as a pivotal player in osteclastogenesis and inflammation-induced bone loss. Here, we discuss our recent results obtained using a genetic approach in mice that indicate the importance of IKKβ, and not IKKα, as a transducer of signals from receptor activator of NF-κB (RANK) to NF-κB. Ablation of IKKβ results in lack of osteoclastogenesis and unresponsiveness of IKKβ-deficient mice to inflammation-induced bone loss. In the need of a more effective therapy for the treatment of inflammatory diseases causing bone resorption, specific inhibition of IKKβ represents a logical alternative strategy to the current therapies.

2. INTRODUCTION

Inflammation is, generally, a beneficial host response to foreign challenge or tissue injury that leads ultimately to restoration of tissue structure and function. In fact, inflammation is an integral part of innate immunity. However, prolonged inflammation that is not resolved ceases to be beneficial and contributes to the pathogenesis of many disease states (Lawrence, Willoughby, et al. 2002). Excessive bone resorption is a major pathological factor in chronic inflammatory diseases such as periodontitis, osteoporosis and arthritis, and it is now clear that disregulation of immune and inflammatory responses is crucial for initiating the bone destruction associated with these conditions (Roodman 1999). The bone-resorbing osteoclasts are known to play a pivotal role in focal bone erosion in rheumatoid arthritis (RA) (Kong, Feige, et al. 1999) and in animal models of arthritis (Gravallese 2002). Studies of tissues obtained from the bone-pannus

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in RA demonstrated the presence of multinucleated osteoclast-like cells (Suzuki, Nishikaku, et al. 1998; Kong, Feige, et al. 1999), and in situ hybridization revealed that these cells expressed mRNA for definitive osteoclast markers such as tartrate resistant acid phospharase (TRAP), cathepsin K, and calcitonine receptor (CTR). In addition, electron-microscopic analysis of subchondral bone from damaged RA metacarpals confirmed the presence of resorption areas typical of osteoclast activity (Leisen, Duncan, et al. 1988). In normal bone physiology osteoclasts differentiate from their hematopoietic precursors and their differentiation is dramatically dependent on osteoblastic/stromal cells of mesenchymal origin that provide a physical support for nascent osteoclasts and produce several soluble and membrane-associated factors that stimulate the proliferation and/or differentiation of hematopoietic osteoclast precursors (Karsenty and Wagner 2002). Imbalances between osteoclast and osteoblast activities can arise from a variety of hormonal changes or perturbations of inflammatory and growth factors, resulting in skeletal abnormalities characterized by decreased (osteoporosis) or increased (osteopetrosis) bone mass (Mostov and Werb 1997; Reddi 1997; Karsenty and Wagner 2002), and the focal net loss of bone in sites of inflammation, as found in RA (Kong, Feige, et al. 1999). In animal models in which expression of key proinflammatory mediators has either been abolished through gene knockouts or their activities modulated through genetic and biochemical blockade, the decrease in the inflammatory response is closely associated with reduction in the degree of bone and cartilage destruction (Ji, Pettit, et al. 2002; Redlich, Hayer, et al. 2002; Shealy, Wooley, et al. 2002; Zwerina, Hayer, et al. 2004). Pettit and colleagues demonstrated that arthritis can be induced in mice lacking osteoclasts (due to the deletion of the key osteoclast differentiation factor, receptor activator of nuclear NF-κB ligand, RANKL), although bone erosion donot occur (Pettit, Ji, et al. 2001). Similar results were obtained in mice lacking the transcription factor c-Fos, which is also required for osteoclast maturation (Redlich, Hayer, et al. 2002). Despite the development and progression of inflammation, these mice were resistant to focal bone erosion as a result of the absence of osteoclasts. Thus, interference with osteoclast formation or maturation represents an attractive strategy for the treatment and prevention of inflammation-induced bone loss, suggesting that blockade of RANKL signaling in combination with an anti-inflammatory cytokine may effects have on both bone erosion and inflammation.

3. THE RANKL-RANK SYSTEM IN PHYSIOLOGICAL AND PATHOLOGICAL BONE REMODELING

Two proteins crucial for osteoclast development and activation are RANK (receptor activator of NF-κB) and its ligand, RANKL. RANKL is a member of the TNF family of cytokines and its expression is regulated by a number of bone resorption-inducing factors including vitamin D3, glucocorticoids, IL-1, IL-6 and TNFα (Nakashima, Wada, et al. 2003) (Romas, Sims, et al. 2002) (Boyle, Simonet, et al. 2003). RANKL activates mature osteoclasts and directs osteoclast differentiation from monocyte/macrophage precursors together with M-CSF (Lacey, Timms, et al. 1998; Boyle, Simonet, et al. 2003). The *in vivo* significance of the RANKL-RANK signaling pathway has been verified by the observations that ablation of either protein in mice results in severe osteopetrosis and a total lack of osteoclasts (Dougall, Glaccum, et al. 1999; Kong, Yoshida, et al. 1999), whereas a deficiency in OPG, which binds to RANK preventing activation of RANK signaling, results in osteoporosis (Simonet, Lacey, et al. 1997). A crucial target of RANKL signaling is transcription factor NF-κB, a finding that implicates this transcription factor in osteoclast differentiation. A critical role for NF-κB in osteoclastogenesis is supported by the fact that gene-specific deletion of both its p50 and p52 subunits causes severe osteopetrosis through the absence of osteoclasts (Iotsova, Caamano, et al. 1997) (Franzoso,

Carlson, et al. 1997). In osteoclasts, RANK induces the activation of Akt, which is blocked by the phosphatidyl inositol 3 kinase (PI3K) inhibitor LY294002 (Lee, Woo, et al. 2002) (Wong, Besser, et al. 1999). Furthermore, LY294002 reduces the RANK-mediated survival response of osteoclasts (Wong, Besser, et al. 1999). The PI3K inhibitor also displays a potent inhibitory effect on osteoclast differentiation (Lee, Woo, et al. 2002), which may result from a reduced survival of osteoclast precursors during differentiation. Although direct evidence for RANK activation of PI3K remains to be demonstrated, it has been shown in osteoclasts that RANK activated the Src tyrosine kinase. The relevance of Src activity to RANK signaling is underscored by the osteopetrotic phenotype of Src-deficient mice (Soriano, Montgomery, et al. 1991). In Src-deficient mice osteoclast motility, and therefore bone resorption, are prevented due to lack of association Src with gelsolin (Chellaiah, Fitzgerald, et al. 1998), thereby inhibiting formation of actin filaments and downregulating the level of Pyk2 and c-Cbl (Duong, Lakkakorpi, et al. 1998) (Tanaka, Amling, et al. 1996).

The RANKL-RANK system also represents a direct link between synovial T-cell infiltration and bone erosion in RA. There is mounting evidence that T lymphocytes regulate osteoclast formation in arthritis. The requirement for RANKL in mediating osteoclast differentiation and function in inflammatory arthritis has been supported by several lines of evidence (Kong, Feige, et al. 1999; Pettit, Ji, et al. 2001; Bolon, Campagnuolo, et al. 2002; Campagnuolo, Bolon, et al. 2002; Redlich, Hayer, et al. 2002; Romas, Sims, et al. 2002; Schett, Redlich, et al. 2003). Initial observations demonstrated that activated T cells provide a source of RANKL for subsequent osteoclast differentiation in rat adjuvant induced arthritis model (Kong, Feige, et al. 1999). RANKL production by activated T cells directly controls osteoclastogenesis and bone remodeling and explains why autoimmune diseases, cancers, leukaemias, asthma, chronic viral infections, and periodontal disease result in systemic and local bone loss (Kong, Feige, et al. 1999). In particular, RANKL seems to be a principle pathogenetic factor that causes bone and cartilage destruction in arthritis. Inhibition of RANKL function via its natural decoy receptor osteoprotegerin (OPG) prevents bone loss in postmenopausal osteoporosis and cancer metastases and completely blocks bone loss and crippling in various rodent models of arthritis. Perhaps, the most important evidence of the crucial role that osteoclasts play in inflammation induced bone loss comes from the study in which OPG was successfully used to block bone erosion in collagen-induced arthritis mice (Kong, Feige, et al. 1999).

4. ROLE OF TNFα IN INFLAMMATION-INDUCED BONE LOSS

Additional cytokines and growth factors that are also produced by cells of the inflamed synovium, primarily the proinflammatory cytokines TNFα and interleukin-1 (IL-1), can also stimulate osteoclast development, thus providing a potential link between the inflammatory process and bone destruction (Kobayashi, Takahashi, et al. 2000) (Lam, Takeshita, et al. 2000) (Zhang, Heulsmann, et al. 2001). Similar to RANKL, TNFα is a potent osteoclastogenic factor that enhances proliferation and differentiation of osteoclast precursors through its type I receptor, TNFR1 (Abu-Amer, Erdmann, et al. 2000) (Zhang, Heulsmann, et al. 2001). However, permissive levels of RANKL are required for optimal TNFα-induced osteoclastogenesis, most likely due to the inability of TNFα to support calcium intake. TNFα mediates RANKL stimulation of osteoclast differentiation through an autocrine mechanism (Zou, Hakim, et al. 2001).

TNFα exerts its biological functions through two receptors: TNFR1 (p55) and TNFR2 (p75). Both receptors are expressed on a wide variety of cell types including bone marrow hematopoietic cells (Sato, Selleri, et al. 1997). Amongst the two receptors it is TNFR1 that mediates most of the biological effects of TNFα, including programmed cell death and the activation of NF-κB

(Karin and Lin 2002). Upon oligomerization, TNFR1 binds to and recruits TNFR-associated death domain protein (TRADD) molecules and binds indirectly to Fas-associated death domain protein (FADD) through an interaction between the death domain of FADD and TRADD. This interaction leads to the activation of a caspase cascade responsible for programmed cell death (Hsu, Shu, et al. 1996). In contrast, TNFR2 lacks a death domain and interacts directly with TRAF2. Although, TRAF2 activates both NF-κB and JNK (Liu, Hsu, et al. 1996), TNFR2 make very little contribution to NF-κB activation (Maeda, Chang, et al. 2003).

Osteoclast recruitment by TNFα is probably essential to the pathogenesis of inflammatory osteolysis. While TNFR1 promotes osteoclastogenesis, TNFR2 was shown to inhibit this process (Abu-Amer, Erdmann, et al. 2000). TNFα is produced primarily by activated T cells and activated macrophages within the inflamed synovial tissue in RA (MacNaul, Hutchinson, et al. 1990; Danning, Illei, et al. 2000). The prominent role of TNFα in driving inflammation has made it a target for biologically-based therapeutics currently used for treatment of RA (Elliott, Maini, et al. 1994; Maini, Breedveld, et al. 1998; Maini, St Clair, et al. 1999; Lipsky, van der Heijde, et al. 2000; Breedveld, Emery, et al. 2004). TNFα alone is sufficient to induce arthritis and joint destruction in a murine model. Mice constitutively expressing human TNFα develop polyarthritis, which is characterized by significant focal bone erosion as well as generalized bone loss (Shealy, Wooley, et al. 2002) (Redlich, Hayer, et al. 2002; Schett, Redlich, et al. 2003). Conversely, mice deficient in TNFα demonstrated a heterogeneous phenotype when challenged with serum transfer arthritis. The absence of TNFα signaling mostly conferred resistance to synovitis and bone erosion, but approximately one-third of the animals studied did develop clinical signs of arthritis, albeit at a delayed rate and reduced severity compared with wild-type littermates (Ji, Pettit, et al. 2002). Furthermore, focal bone erosion, correlating roughly with the degree of inflammation, was evident within the affected joints in the animals that did develop clinical arthritis (Ji, Pettit, et al. 2002). This study supported previous observations in which TNFα-deficient mice were subject to collagen-induced arthritis (Campbell, O'Donnell, et al. 2001), suggesting that TNFα-independent pathways can compensate for the loss of TNFα signaling in mediating inflammation and subsequent bone erosion (Campbell, O'Donnell, et al. 2001; Ji, Pettit, et al. 2002). TNFα antagonists, either alone (Moreland, Baumgartner, et al. 1997; Moreland, Schiff, et al. 1999; Bathon, Martin, et al. 2000; Genovese, Bathon, et al. 2002) or in combination with the immunosuppressant methotrexate (Elliott, Maini, et al. 1994; Maini, Breedveld, et al. 1998; Maini, St Clair, et al. 1999; Weinblatt, Kremer, et al. 1999; Lipsky, van der Heijde, et al. 2000; den Broeder, van de Putte, et al. 2002; den Broeder, Joosten, et al. 2002; Kremer, Weinblatt, et al. 2003; Weinblatt, Keystone, et al. 2003; Breedveld, Emery, et al. 2004) have demonstrated efficacy in reducing signs and symptoms of RA and arrest progression of erosions in a large number of RA patients. Despite its apparent efficacy, it is not effective in all the patients. Some of them, indeed, do not respond to anti-TNFα therapy, and complete disease remission, including the prevention of bone loss, is not always achieved. This suggests that, as in experimental arthritis models, alternate pathways that mediate inflammation and bone erosion may contribute to the heterogeneity of disease and that in cases in which TNFα blockade is insufficient to control the disease process, alternative therapeutic strategies need to be considered.

5. NF-κB AND IKKβ AS REGULATORS OF INFLAMMATION AND BONE REMODELING

NF-κB activity is regulated through interaction with specific inhibitors, IκBs, which trap NF-κB dimers in the cytoplasm (Ghosh, May, et al. 1998). In response to cell stimulation with proinflammatory and innate immune stimuli, such as TNFα, IL-1, or bacterial

endotoxin (LPS), the IκBs are phosphorylated at two conserved serines and targeted to rapid ubiquitin-dependent proteolysis (Karin and Ben-Neriah 2000). IκB phosphorylation is carried out by the IκB kinase (IKK), a complex composed of three subunits: IKKα, IKKβ, and IKKγ/NEMO (Rothwarf and Karin 1999). IKKα and IKKβ serve as the catalytic subunits, whereas IKKγ/NEMO is the regulatory subunit. IKKα and IKKβ contain very similar kinase domains with essentially identical activation loops (Mercurio, Zhu, et al. 1997; Zandi, Rothwarf, et al. 1997). Despite their structural and biochemical similarities, IKKα and IKKβ are functionally distinct (Rothwarf and Karin 1999). Whereas IKKβ is essential for NF-κB activation in response to proinflammatory and innate immune stimuli, IKKα is not required for such responses (Hu, Baud, et al. 1999; Li, Van Antwerp, et al. 1999; Li, Chu, et al. 1999; Senftleben, Li, et al. 2001). IKKα, however, plays a unique and critical role in development of the epidermis (Hu, Baud, et al. 1999), but its ability to induce keratinocyte differentiation is independent of its protein kinase activity or NF-κB (Hu, Baud, et al. 2001). Recently, IKKα was found to be required for B cell maturation, another unique function that is not provided by IKKβ (Senftleben, Cao, et al. 2001). This function is dependent on IKKα kinase activity, but instead of being mediated through inducible IκB degradation, is exerted via a second NF-κB activation pathway, dependent on processing of the NF-κB2/p100 precursor protein to the mature p52 subunit (Senftleben, Cao, et al. 2001). This pathway requires the activity of another protein kinase, NIK (NF-κB inducing kinase), which may function as an activator of IKKα (Senftleben, Cao, et al. 2001). It was observed that NIK-deficient osteoclast precursors do not respond to RANKL in an *in vitro* differentiation system devoid of osteoblasts (Novack, Yin, et al. 2003). However, *aly* mice, which carry a point mutation in the *Nik* gene that prevents NIK activation, are not osteopetrotic (Shinkura, Kitada, et al. 1999), neither was osteopetrosis reported for $Nik^{-/-}$ mice (Novack, Yin, et al. 2003). In addition, we found that a mutation that prevents IKKα activation has no effect on bone development or inflammation-induced bone loss *in vivo* (Ruocco, Maeda, et al. 2005). Nonetheless, a pivotal role for transcription factor NF-κB in regulation of inflammation has been well recognized (Barnes and Karin 1997; Tak and Firestein 2001). As mentioned above, the relevance of NF-κB pathway to osteoclastogenesis is underscored by the osteopetrotic phenotypes of mice lacking the NF-κB1/p50 and NF-κB2/p52 subunits (Franzoso, Carlson, et al. 1997; Iotsova, Caamano, et al. 1997). Interestingly, a deficiency in a single subunit has no effect on osteoclast formation or maturation. The question, however, is which catalytic subunit is required for NF-κB activation during osteoclastogenesis and inflammation-induced bone loss.

NF-κB controls the expression of the proinflammatory cytokines IL-1β and TNFα, which are important mediators of inflammation in RA. In turn, both TNFα and IL-1β are potent inducers of NF-κB activation, suggesting an interdependence of persistent NF-κB activation and sustained IL-1β and TNFα production. Indeed, expression of a non-phosphorylatable variant of the NF-κB inhibitor IκBα (srIκBα) abrogated the induction of IL-1β and TNFα in human macrophages and primary fibroblast-like sinoviocytes (FLS) (Miagkov, Kovalenko, et al. 1998) (Foxwell, Browne, et al. 1998). More importantly, a small synthetic peptide that disrupts the interaction between IKKβ and the IKKγ regulatory subunit and therefore prevents IKK activation was found to inhibit inflammation-induced bone loss in a mouse model of arthritis (Jimi, Aoki, et al. 2004).

A recent study from our laboratory established that IKKβ, but not IKKα, is essential for inflammation-induced bone loss and is required for osteoclastogenesis *in vivo* (Ruocco, Maeda, et al. 2005). IKKβ-deficient BM cells do not form osteoclasts *in vitro* when stimulated with RANKL.

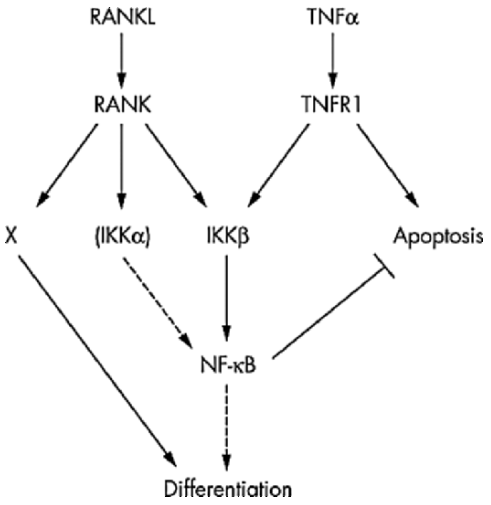

Figure 1. Schematic model of receptor activator of nuclear factor (NF)-κB ligand (RANKL) and tumour necrosis factor a (TNFα) signaling during osteoclastogenesis and inflammation induced bone loss. X, a pathway other than IκB kinase (IKK)/NF-κB that is activated by RANKL binding to RANK and is essential for production of functional osteoclasts. IKKα function in RANK signalling is dispensable. TNFR1, TNF receptor 1.

Furthermore, mice lacking IKKβ in hematopoietic cells and hepatocytes, *Ikkβ*$^{\Delta}$ mice, are osteopetrotic, due to the lack of osteoclasts, indicating that IKKα function is dispensable *in vivo* in RANK signaling pathway (Figure 1).

However, the main function of IKKβ in osteoclastogenesis is to prevent TNFα-induced apoptosis of osteoclast precursors (Figure 1). Indeed, IKKβ-deficient BM cells are extremely sensitive to TNFα-induced apoptosis and die in response to elevated TNFα. Loss of TNFR1 prevents apoptosis in IKKβ-deficient BM cells and restores the presence of osteoclasts in mice that lack both IKKβ and TNFR1 in the relevant cells, *Ikkβ*$^{\Delta}$/*Tnfr1*$^{-/-}$ mice. Nonetheless, the prevention of TNFα-induced death reveals that IKKβ is also required for maturation of functional osteoclasts, because *Ikkβ*$^{\Delta}$/*Tnfr1*$^{-/-}$ osteoclasts are defective in bone resorption. Inflammation-induced bone loss is prevented in mice lacking IKKβ, because IKKβ-deficient osteoclasts and preosteoclasts are killed by TNFα. Once the effect of TNFα is eliminated by ablation of its receptor, inflammation-induced bone loss is restored in *Ikkβ*$^{\Delta}$/*Tnfr1*$^{-/-}$ mice (Ruocco, Maeda, et al. 2005). Thus, despite the inability of IKKβ and TNFR1-deficient osteoclasts to undergo functional maturation (i.e. become active in bone resorption) under non-inflamed condition, in the presence of a strong inflammatory stimulus, such as the one generated by LPS-injection into the joint, these cells undergo maturation after all (Ruocco, Maeda, et al. 2005). The inflammatory cytokines that induce the functional maturation of *Ikkβ*$^{\Delta}$/*Tnfr1*$^{-/-}$ osteoclast precursors remain to be identified.

6. THERAPEUTIC INTERVENTIONS

Knowledge of the pathogenic mechanisms of inflammatory arthritis has led to the design of targeted therapies that are effective in suppression of inflammation and prevention of joint destruction. The current anti-inflammatory and antirheumatic drugs used to treat rheumatoid

arthritis include glucocorticoids, aspirin, sodium salicylate, sulfosalazine and gold compounds, all of which have been shown to block NF-κB activity (Epinat and Gilmore 1999). The list of therapeutics that inhibit NF-κB also includes numerous natural and synthetic antioxidants, immunosuppressants, and natural plant compounds, suggesting that the ability to suppress NF-κB activation at least partially accounts for their therapeutic effects (Epinat and Gilmore 1999). These compounds are neither potent nor selective for this pathway, however, and may have a range of undesirable side effects as a result of their nonspecific nature. Consequently, response to treatment in RA patients is not always complete, and in a subset of patients, focal bone erosion progresses despite therapy. More clear answers were obtained using animals with genetically inactivated NF-κB signaling. Ablation of the *nf-κb1* and *rel* genes rendered the affected animals refractory to development of collagen-induced arthritis (CIA) (Campbell, Gerondakis, et al. 2000). Transgenic mice expressing a super-repressor form of IκBα (srIκBα) in the T lineage were similarly refractory to CIA (Seetharaman, Mora, et al. 1999). These genetic studies are in a good agreement with the experiments that utilized highly specific inhibitors of NF-κB. However, the safety of a long-term use of specific NF-κB inhibitors remains to be elucidated. Genetic studies revealed that NF-κB activity is required for provision of innate immunity and prevention of opportunistic infections (Bonizzi and Karin 2004). Highly specific inhibitors, local delivery, and short-term treatments should alleviate the possible side effects associated with systemic inhibition and minimize the risk of general immunosuppression.

In this context, the results from our knockout experiments (Ruocco, Maeda, et al. 2005) and those obtained by the use of IKK peptide inhibitor (Jimi, Aoki, et al. 2004) are exciting. They indicate that specific and selective inhibition of the IKKβ subunit and the classical NF-κB activation pathway represent an effective approach to the treatment of inflammatory diseases causing bone resorption. Furthermore, during inflammation, proinflammatory cytokines such as TNFα and IL-1β are induced and strongly potentiate RANKL-induced osteoclastogenesis, although such factors can not induce osteoclast differentiation on their own (Ruocco, Maeda, et al. 2005) (Lam, Takeshita, et al. 2000). TNFα signaling through TNFR1 has the potential to induce apoptosis through caspase 8, a process that is prevented by IKKβ-dependent NF-κB activation (Karin and Lin 2002). Once IKKβ is inhibited, TNFα-induced apoptosis can eliminate *Ikk*β-deficient osteoclast progenitors, thereby preventing inflammation-induced bone destruction. Thus, IKKβ inhibition presents a logical strategy for the therapy of numerous bone-resorbing inflammatory disorders in which TNFα is elevated, such as rheumatoid arthritis. However, the efficacy of such an approach would be severely compromised if TNFα signaling, responsible for the elimination of IKKβ-inhibited osteoclast progenitors, is blocked by anti-TNFα drugs, such as infliximab and etanercept. Thus, when IKKβ inhibitors will become available it is unlikely that they will be useful in conjunction with the currently available anti-TNFα therapeutics.

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CHAPTER 17
TARGETING OSTEOPOROSIS AND RHEUMATOID ARTHRITIS BY ACTIVE VACCINATION AGAINST RANKL

Gunther Spohn and Martin F. Bachmann*

1. INTRODUCTION

Chronic diseases like osteoporosis and rheumatoid arthritis (RA) represent a major burden for the society, both in terms of quality of life for the affected individuals and associated health care costs. Approximately 25–30% of postmenopausal women are affected by osteoporosis, and RA occurs in about 1% of the adult population worldwide, with numbers expected to rise substantially as the average life expectancy in western societies increases (Riggs, Khosla, and Melton 2002; Lawrence, Helmick, Arnett, et al. 1998). Conventional treatments – mainly bisphosphonates and selective estrogen modulators in the case of osteoporosis, and NSAIDs and DMARDs in the case of RA - are efficient, but dosing regimens are sometimes difficult and side effects are often considerable. Novel therapeutic approaches are aimed at blocking specifically the molecular interactions at the basis of the disease process. For the treatment of RA a number of monoclonal antibodies (mAbs) and other biologicals are now available which neutralize central inflammatory cytokines like TNFα or IL-1 or lead to depletion of disease-promoting B cells. Likewise, a mAb specifically targeting the RANK-RANKL interaction is in development for the treatment of osteoporosis and has shown very promising results (McClung, Lewiecki, Cohen, et al. 2006). Although highly efficient, these biological therapies have several potential drawbacks, including the high cost of goods and the risk of generating neutralizing antibody responses, which might limit their long term efficacy. Expensive production of mAbs has important economical implications, since it often prevents the use of such drugs as first-line treatment. A point in case are mAbs directed against TNFα; due to their high production costs they cannot compete with e.g. methotrexate financially; consequently, such drugs cannot easily become first line treatment and mass products. This is particularly unfortunate in this case, since TNFα-blocking agents are the most important disease modifying drugs for the treatment of RA. Although the frequency of administration may be lower for mAbs directed against

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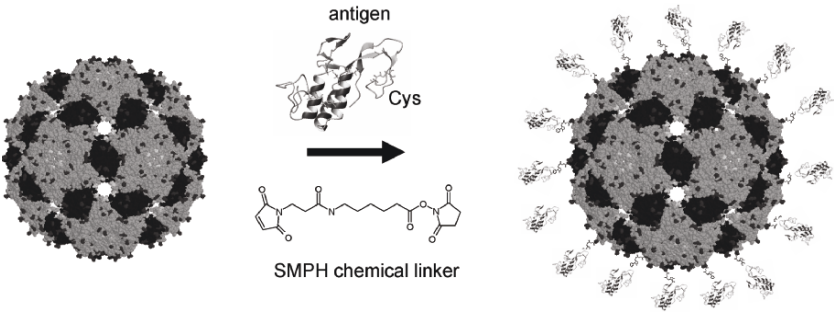

Figure 1. Modular assembly system for the generation of autologous VLP vaccines. The auto-antigen of choice is recombinantly expressed with an additional cysteine residue at its N- or C-terminus and chemically attached via a heterobifunctional cross-linker (e.g. SMPH = Succinimidyl-6-[ß-maleimidopropionamido]hexanoate) to lysine residues on the surface of virus-like particles of a bacteriophage. The resulting vaccine displays a high number of self-antigens in an ordered and highly repetitive fashion on its surface, leading to strong and sustained auto-antibody production.

RANKL (McClung, Lewiecki, Cohen, et al. 2006; Bekker, Holloway, Rasmussen, et al. 2004), the problem remains similar and it will be difficult to treat osteoporosis patients early, before onset of severe symptoms, with such expensive biologicals. Hence, a need for convenient, disease modifying and cost-effective long term treatments for such chronic diseases still exists.

Induction of auto-antibodies against disease-associated molecules by active immunization might be such a cost-effective alternative to the administration of mAbs. We have previously shown that by displaying self-antigens in a highly repetitive fashion on the surface of virus-like particles (VLPs), the natural tolerance of the immune system towards these antigens can be broken and a strong and long-lasting auto-antibody response can be achieved (Jegerlehner, Tissot, Lechner, et al. 2002; Lechner, Jegerlehner, Tissot, et al. 2002). In the system developed by us the self antigen is recombinantly expressed with an additional cysteine residue at its N- or C-terminal end and covalently attached via a bifunctional cross-linker to lysine residues on the viral coat protein forming the VLP (Figure 1). In this way a large number of antigen molecules can be displayed on the VLP in an oriented and highly repetitive fashion, leading to efficient cross-linking of B cell receptors and sustained antibody production. Similar systems have been developed by others where self-antigens are fused to VLP coat proteins or displayed on the VLP-surface by means of biotin-avidin interactions (Chackerian, Lowy, and Schiller 2001).

2. AN AUTOLOGOUS VACCINE AGAINST RANKL

In an attempt to produce a therapeutic vaccine against postmenopausal osteoporosis, a modified version of the extracellular domain of murine RANKL containing a cysteine residue at its N-terminus was purified and rendered highly repetitive and organized by covalent attachment to virus-like particles of the bacteriophage Qβ. These VLPs form capsids with a diameter of ~30 nm and consist of 180 subunits. Analysis of the conjugate Qβ-RANKL vaccine revealed that about 25 RANKL trimers were displayed per VLP, indicating that about half of the surface of the VLP was covered by RANKL. Immunization of mice with 25 µg of this conjugate vaccine without the addition of any adjuvant yielded high titers of RANKL-specific auto-antibodies. Additional injections enhanced the response against RANKL, while specific antibody titers

dropped in the absence of further injections. The half-life of the response was reproducibly in the range of 2–3 months (Spohn, Schwarz, Maurer, et al. 2005).

Although we have not immunized yet humans with a vaccine against RANKL, we have done so with a vaccine against TNFα, in which a TNFα-derived peptide was displayed on Qβ VLPs. As observed in the mouse for RANKL, vaccinated volunteers developed specific antibody titers against TNFα. The antibody titers increased with booster injections and declined with a relatively fast half-life in the range of weeks to months in the absence of further immunizations (unpublished). Thus, humans and mice appear to respond similarly to self-antigens displayed on virus-like particles.

Whether vaccination against RANKL resulted in neutralizing antibodies was assessed by competition ELISA. As expected, RANKL-specific antibodies elicited by vaccination efficiently blocked the interaction of RANK with RANKL (Spohn, Schwarz, Maurer, et al. 2005). The antibodies were also tested for their ability to inhibit the formation of osteoclasts from bone marrow cells *in vitro*. Again, the anti-serum potently inhibited the action of RANKL, since osteoclast formation was strongly reduced.

3. EFFICACY IN OSTEOPOROSIS

In order to test the efficacy of our autologous vaccination approach in the prevention of osteoporosis, mice were immunized with Qβ-RANKL and subjected to complete ovariectomy to induce estrogen depletion and initiate bone resorption, and compared to non-immunized ovariectomized or sham operated controls. Five weeks after ovariectomy, serum markers of bone resorption (Figure 2, panel A) and bone formation (Figure 2, panel B) were significantly increased in the non-immunized ovariectomized control group. Qβ-RANKL-immunized ovariectomized mice on the other hand displayed similar levels to the sham operated group, indicating that the RANKL-specific antibodies had blocked the increase in bone turnover induced by the ovariectomy (Figure 2, panels A, B). Measurement of different bone parameters in the cortical region of the femurs after sacrifice of the animals confirmed the protective effect of Qβ-RANKL immunization on bone structure. The strong loss of overall bone mineral density observed in ovariectomized control animals was almost completely prevented by anti-RANKL vaccination (Figure 2, panel C) and the decrease in cortical thickness caused by enhanced endosteal resorption was strongly inhibited (Figure 2, panel D). Histomorphometric analysis of the area of secondary spongiosa revealed, that the trabecular architecture had been essentially conserved by vaccination against RANKL. The overall trabecular bone volume (Figure 2 panel E) and the trabecular number (Figure 2 panel F) as well as the mean trabecular thickness and separation (not shown) were similar in sham operated animals and Qβ-RANKL-immunized ovariectomized animals. Taken together, these data indicate that active immunization against RANKL might represent an efficient treatment of postmenopausal osteoporosis.

4. EFFICACY IN RHEUMATOID ARTHRITIS

The role of the RANKL/RANK system as a critical player in the accelerated osteoclastogenesis and bone resorption seen in inflamed joints of RA patients has been well established. It has been shown that activated T cells and synoviocytes, which are present in arthritic joints, produce large amounts of RANKL and M-CSF, which induce the differentiation of osteoclast precursors into mature bone-resorbing osteoclasts (Kong, Yoshida, Sarosi, et al. 1999; Kong, Feige, Sarosi, et al. 1999; Gravallese, Manning, Tsay, et al. 2000; Takayanagi, Iizuka,

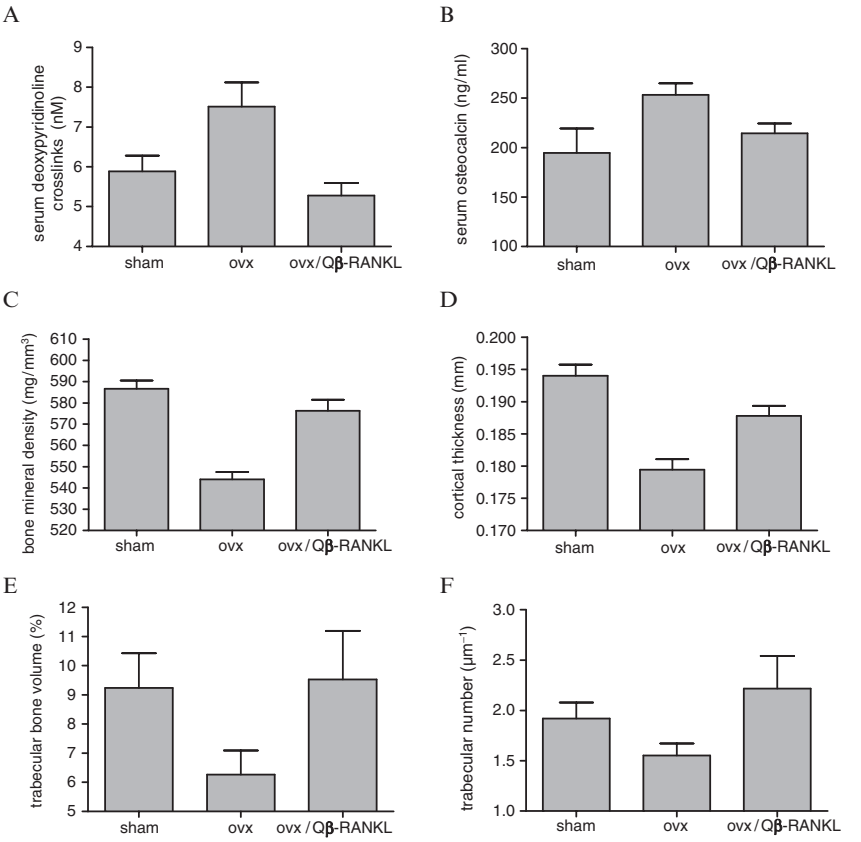

Figure 2. Active immunization against RANKL protects from ovariectomy-induced osteoporosis. Female Balb/c mice were immunized four times on days 0, 14, 21, and 42 with 50 µg Qβ-RANKL, and subjected to complete ovariectomy on day 35 (ovx / Qβ-RANKL). At the same time two non-immunized age-matched control groups were subjected to either ovariectomy (ovx) or sham operation (sham). (A, B) Biochemical markers of bone degradation (deoxypyridinoline cross-links) and bone formation (osteocalcin) were measured in serum five weeks after ovariectomy and revealed a reduction of osteoblast and osteoclast activities in animals immunized against RANKL. (C, D) Analysis of the cortical area of mouse femurs by pQCT measurements after sacrifice revealed a strong protective effect of anti-RANKL vaccination on bone mineral density and cortical thickness. (E, F) Histomorphometric analysis of the trabecular area of femurs showed a strong conservation of the trabecular architecture by immunization with Qβ-RANKL.

Juji, et al. 2000). The role of RANKL in the pathogenesis of the inflammatory process itself has been discussed much more controversially. Early studies using different rodent models for rheumatoid arthritis had suggested that RANKL signalling is essential for inflammation-driven osteoclastogenesis, but not for development of the inflammation itself. For example Pettit and coworkers had shown that transfer of arthritogenic serum from KBxN mice to RANKL$^{-/-}$ mice lead to reduced bone destruction but caused normal levels of inflammation (Pettit, Ji, von Stechow, et al. 2001). Similarly, treatment of Lewis rats with an OPG-Fc fusion molecule before induction of arthritis prevented bone loss but had no influence on the inflammatory response (Kong, Feige, Sarosi, et al. 1999). More recent studies however suggest that RANKL signalling is indeed involved in controlling inflammation. Seshasayee and coworkers have shown that

treatment of isolated human peripheral monocytes with soluble RANKL induces the release of the proinflammatory cytokines TNFα, IL-1β, IL-6 and IL-12, and that administration of a RANK-Fc fusion molecule protects mice from clinical and histological manifestations of inflammation in an anti-collagen-antibody-induced arthritis model (Seshasayee, Wang, Lee, et al. 2004). Using an IL-2-deficient mouse model of autoimmunity, it has further been shown that administration of a recombinant version of osteoprotegerin, the decoy receptor of RANKL, can reduce not only osteopenia, but also intestinal inflammation (Ashcroft, Cruickshank, Croucher, et al. 2003).

We decided to test the effect of Qβ-RANKL immunization on the inflammatory response in the murine collagen-induced arthritis model. We used two different experimental settings, a prophylactic one, where mice were immunized with Qβ-RANKL before the disease was induced, and a therapeutic setting, where purified IgG raised against Qβ-RANKL was passively administered after disease induction. As shown in Figure 3 (panel A), immunization of mice before disease induction strongly protected from clinical signs of arthritis, indicating that blocking RANKL signaling during the priming phase has a strong influence on the magnitude of the inflammatory response. Interestingly, also administration of RANKL-specific IgG after disease onset reduced disease symptoms, suggesting that the RANKL/RANK system has an important role also during the effector phase of the disease (Figure 3, panel B).

Although the precise nature of the inflammatory effector cells which are activated by RANKL signalling in this model remains to be elucidated, it is likely that circulating osteoclast precursors play an important role. The numbers of these $CD11b^{+}/Gr\text{-}1^{-/lo}$ cells have been shown to be increased in 4 month-old TNFα-transgenic mice with established arthritis, but not in healthy young transgenic mice (Li, Schwarz, O'Keefe, et al. 2004). When stimulated with RANKL these cells produce high amounts of TNFα (Xing, Schwarz, and Boyce 2005). Crossing of TNFα transgenic mice with $RANK^{+/-}$ mice on the other hand leads to TNF-Tg X $RANK^{-/-}$ hybrids which have reduced systemic TNFα concentrations and completely lack not only osteoclasts but also joint inflammation (Li, Schwarz, O'Keefe, et al. 2004). The role of the pool of osteoclast precursors in the collagen-induced arthritis model has yet to be elucidated;

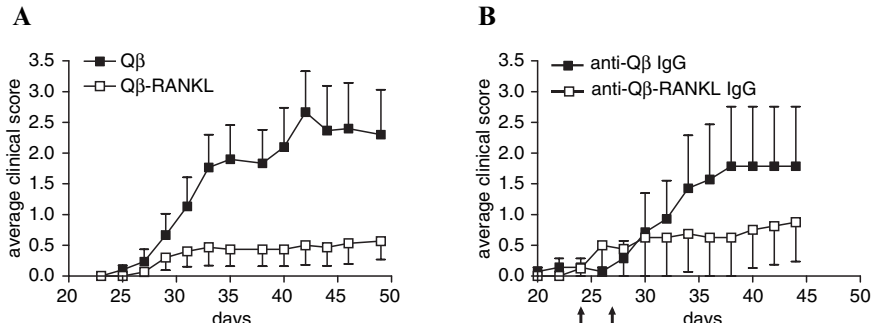

Figure 3. Active immunization against RANKL protects from collagen-induced arthritis. (A) Male DBA/1 mice were immunized three times at two week intervals with 50 µg Qβ-RANKL or Qβ VLPs alone and then injected with collagen/CFA (d0) and collagen/IFA (d21) to induce arthritis. (B) Male DBA/1 mice were injected with collagen/CFA (d0) and collagen/IFA (d21) to induce arthritis and then injected intraperitoneally on days 24 and 27 (arrows) with 500 µg purified murine IgG, which had been raised against either Qβ-RANKL or Qβ VLPs alone. Development of disease symptoms was observed in regular intervals and a clinical score ranging from 0–3 was assigned to each limb, resulting in a maximal cumulative score of 12 per mouse. Represented are the averages of the individual mouse scores with standard errors of the mean.

it has, however, been shown that IFNγR$^{-/-}$ mice, which develop earlier and more severe disease symptoms in the collagen-induced arthritis model than wild type mice, have an increased number of CD11b^{+} osteoclast precursors in the spleen, indicating a role for this cell type in promoting the inflammatory response (De Klerck, Carpentier, Lories, et al. 2004).

5. SAFETY CONSIDERATIONS

How does the safety and tolerability of a vaccine against RANKL compare to mAbs? Several distinct mechanisms may be responsible for adverse reactions to the vaccine or the mAbs. Both drugs may cause local or systemic problems within a few hours after injection (acute toxicity), side-effects may occur due to neutralization of RANKL and the vaccination approach may cause an uncontrollable autoimmune response against RANKL. Acute toxicity of vaccines is generally low and may include local side-reactions as well as flu-like symptoms. Severe side-reactions may not be expected. In contrast, mAbs may be able to cause severe systemic anaphylactic reactions in sensitized individuals, in particular if administered intravenously. Hence, depending on the final product and formulation, mAbs may be able to cause severe side-reactions, requiring in some cases medical supervision during administration. Both injection of mAbs and vaccination would result in neutralization of RANKL activity. Since RANKL plays only a limited role in protection from infection (Bachmann, Wong, Josien, et al. 1999) and does not appear to have many functions in addition to the regulation of bone formation and activation of innate cells, its neutralization may be expected to be a relatively safe manipulation of the patient's body. Furthermore, since RANKL-specific antibody titers induced by active vaccination are expected to drop with kinetics not too dissimilar from mAbs, the extent of the problem may be similar for both drugs. The same is true for the formation of immune complexes, which will be similar for vaccine-induced and monoclonal Abs (in particular since RANKL is a trimer). Antibody-dependent cellular cytotoxicity (ADCC) may also not differ for the two types of antibodies and in fact we have found no evidence for ADCC in our preclinical models. Induction of an uncontrolled immune response against RANKL is finally a risk only associated with the vaccine. As discussed above, we have seen reversible antibody responses against RANKL in mice and against similar proteins in humans. Hence, uncontrolled B cell responses do not appear to be induced normally. This may be explained by the fact that B cells only proliferate and differentiate to antibody producing plasma cells in response to antigen plus specific T cell help. Since vaccination does not result in RANKL-specific T helper cells, specific B cells are only stimulated by endogenous RANKL alone and are therefore not sufficiently activated to mount an antibody response. This is different during the vaccination process, where B cells are additionally stimulated by VLP-specific T helper cells (for review see Bachmann and Dyer (2004)).

Taken together, these considerations suggest that the safety profile of a vaccine against RANKL is relatively similar to the one of a RANKL-specific mAb; if carefully performed, the development of such a vaccine should therefore be feasible.

6. COSTS OF GOOD FOR MONOCLONAL ANTIBODIES VERSUS VACCINATION

As mentioned above, high costs of good of monoclonal antibodies may hinder their wide-spread use, in particular in patients at an early stage of the disease. Is this different for VLP-based vaccines? Table 1 estimates quantities of vaccines versus mAbs needed for therapy as well as production volumes required for their generation.

Table 1. Estimated differences in productivity profiles and dosing regimens between monoclonal antibodies and VLP-based vaccines.

	VLP-based vaccine	MAb	Factor
Productivity per liter	0.5–2 g	0.4 g	1.25 – 5
Production batches	2/week	1/month	8
Dose per administration	100 µg	50 mg	500
Frequency of administration	2–4/year	2–24/year	1–6
Overall efficiency gain			5'000–120'000

If productivity and dose of administration is taken into account, an overall efficiency gain of 5′000–120′000 emerges. Hence, from a cost of good perspective, a vaccine against RANKL could be offered at a much more competitive price than mAbs. As a consequence, were such a vaccine proven to be safe, it could be used as a first-line treatment, halting the disease before onset of symptoms.

The human population is rapidly ageing in both the developed as well as the developing world. Treating chronic diseases in the elderly therefore becomes more and more of a challenge and a financial burden for healthcare systems as well as our society in general. A vaccine against RANKL which is both disease-modifying as well as cost-effective may therefore offer a new possibility for the prevention and/or treatment of osteoporosis and RA throughout the population both in the developed and the less developed world.

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CHAPTER 18
RANKL INHIBITION: FROM MICE TO MEN (AND WOMEN)

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1. ABSTRACT

RANKL, the primary mediator of osteoclast formation, function and survival, is implicated in bone loss across a broad range of conditions. RANK and RANKL are expressed by cells involved in bone remodeling, by cells of the immune system, and by cells in other tissues. Preclinical and clinical data support the following conclusions: 1) The immune and skeletal phenotypes associated with RANKL inhibition differ in important ways from those associated with the complete absence or ablation of RANK or RANKL. 2) Immune challenge performed in animals in the presence of RANKL inhibition demonstrates normal immune function, consistent with the interpretation that RANKL inhibition does not impair the ability of animals to mount an effective immune response. 3) In animal models of inflammatory disease, inhibition of RANKL prevents bone loss but does not show a detectable effect on immune mediators or inflammation. 4) A phase 2 study in postmenopausal women with low BMD using the RANKL inhibitor denosumab showed an increase in BMD with an incidence of adverse events that was similar to placebo and to open-label alendronate. In addition, in a subset of patients tested for immunological markers, there were no clinically meaningful differences in T, B, or NK cell numbers or in immunoglobulin levels across dose or treatment groups.

2. INTRODUCTION

The signaling pathway that is activated upon receptor activator of NF-kappa B ligand (RANKL) binding to its receptor RANK is central to bone remodeling (Boyle, Simonet, and Lacey 2003). RANKL is produced by osteoblasts, stromal cells, and other cells and is an essential

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mediator of osteoclast formation, function, and survival (Fuller, Wong, Fox, et al. 1998; Lacey, Timms, Tan, et al. 1998; Yasuda, Shima, Nakagawa, et al. 1998; Lacey, Tan, Lu, et al. 2000; Boyle, Simonet, and Lacey 2003). RANK is expressed on mature osteoclasts and their precursors. RANKL binding to RANK results in increased formation, function, and survival of osteoclasts, leading to increased bone-resorbing activity. Osteoprotegerin (OPG) is an endogenous inhibitor that binds to and prevents RANKL from binding RANK, thereby reducing osteoclast activity.

The essential requirement of RANKL and RANK in bone resorption was demonstrated using knockout animals. Mice lacking RANK or RANKL are unable to produce mature osteoclasts and are born with osteopetrosis. Transgenic mice overexpressing the RANKL inhibitor, OPG, have osteosclerosis, but in contrast to the knockout, have normal tooth eruption and normal bone shape (Simonet, Lacey, Dunstan, et al. 1997; Dougall, Glaccum, Charrier, et al. 1999; Kong, Yoshida, Sarosi, et al. 1999). Mice lacking OPG are severely osteopenic due to unopposed RANKL action driving bone resorption (Bucay, Sarosi, Dunstan, et al. 1998; Mizuno, Amizuka, Irie, et al. 1998).

There are many tissues in which RANK or RANKL mRNA is expressed. In the immune system, RANKL is expressed by activated T and B cells, whereas expression of RANK is mostly confined to mature dendritic cells—cells which share a common lineage with osteoclasts and macrophages (Anderson, Maraskovsky, Billingsley, et al. 1997; Wong, Josien, Lee, et al. 1997; Choi, Woo, Ko, et al. 2001). Although these molecules are expressed, a functional and non-redundant role in the adult immune system has yet to be identified.

3. INHIBITION VS ABSENCE OF RANKL

Inhibition of the RANKL pathway (as with OPG administration or overexpression) differs from complete absence or ablation of RANKL or RANK molecules, as shown by the phenotypes of transgenic (RANKL inhibition) and knock-out (ablation of RANK or RANKL) animals. Ablation of the RANKL or RANK genes in mice results in absence of lymph nodes (Dougall, Glaccum, Charrier, et al. 1999; Kong, Yoshida, Sarosi, et al. 1999). The interaction of RANK and RANKL appears to be an essential mediator of the differentiation of lymph node 'inducer' cells during embryogenesis of the lymph node anlagen in mouse at approximately fetal day 14 (Mebius 2003). Altered development and differentiation of lymphocytes, absence of tooth eruption, osteopetrosis, and absence of lactation are also seen in these animals (Kong, Yoshida, Sarosi, et al. 1999). Animals with the absence of RANK or RANKL have normal Peyer's patches, normal splenic architecture, and normal dendritic cell numbers and function, suggesting that RANK/RANKL is not essential for formation of these organs or for dendritic cell development or function.

In contrast, inhibition of this pathway by transgenic overexpression of OPG in mice does not affect lymph node formation or lymphocyte function (Simonet, Lacey, Dunstan, et al. 1997). Thus, the phenotype of RANKL inhibition is very different from that of RANKL absence in terms of embryogenesis of the immune system. In addition, as discussed below, numerous studies with exogenous RANKL inhibitors support the idea that RANKL inhibition in adult animals does not affect the immune system.

4. RANKL INHIBITION AND IMMUNE CHALLENGE

Immune challenges performed in animals in the presence of RANKL inhibition demonstrate normal immune function, consistent with the interpretation that the RANKL/RANK pathway is not essential for an animal to mount a normal immune response. In studies of mice

treated repeatedly with OPG, RANKL inhibition did not affect cell-mediated reactions such as contact hypersensitivity or granuloma formation, innate immunity, or humoral responses to defined immune challenges (Stolina, Guo, Faggioni, et al. 2003). For example, as seen in Figure 1, in a contact hypersensitivity model (testing the T cell–dendritic cell interaction), mice treated with OPG-Fc or Fc alone exhibited no detectable difference in extent of ear swelling or in the kinetics of the response. In contrast, administration of CTLA-4, a negative regulator of T-cell activation, showed markedly reduced contact hypersensitivity.

Response to viral infections such as influenza has been investigated in other studies. Mice were given influenza virus after being treated with either placebo, RANK:Fc (a RANKL inhibitor), or dexamethasone. The animals treated with RANK:Fc were indistinguishable from those treated with placebo in terms of viral clearance and in anti-influenza antibody production, whereas the dexamethasone group was significantly slower to clear virus and produced significantly less anti-influenza IgG (Miller, Branstetter, Jones, et al. 2005).

A few published accounts describe immunomodulatory effects of RANKL inhibition. However, in the models studied, major immunologically active molecules (eg, CD40 or IL-2) are completely absent, and alternative pathways such as RANKL/RANK may be needed to provide at least some backup to the CD40/CD40L interactions normally required between T-cells, B-cells, and dendritic cells (Bachmann, Wong, Josien, et al. 1999). For example, when the CD40 molecule was absent as in CD40-knockout mice, stimulation of the RANKL/RANK pathway

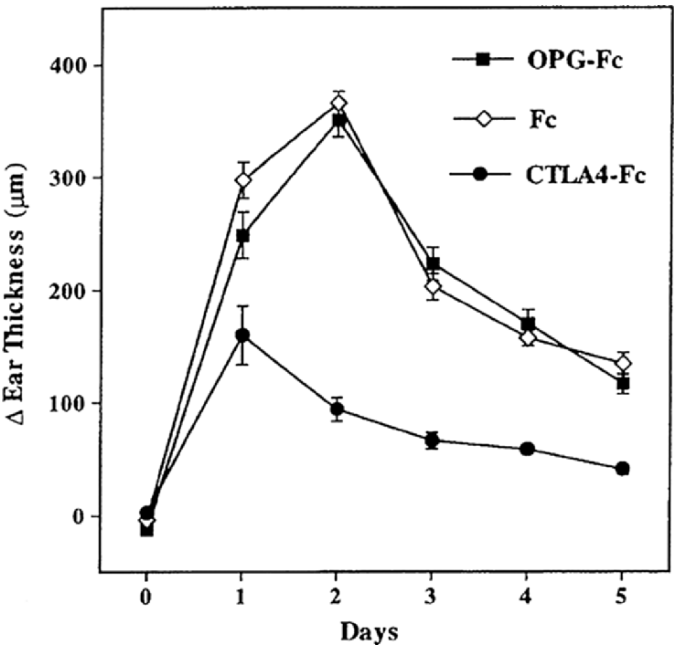

Figure 1. Effects of OPG on contact hypersensitivity. Mice were challenged with oxazolone 7 days after sensitization (day 0). Mice (n = 10/group) were treated with OPG-Fc, CTLA4-Fc, or Fc alone as a control (5 mg/kg) every other day beginning the day before sensitzation until day 4 after challenge. OPG-Fc treatment and Fc treatment showed similar increases in ear thickness (ΔEar Thickness); CTLA4-Fc significantly decreased ear swelling (ΔEar Thickness) compared with OPG-Fc or Fc at all time points ($P < 0.001$). Reprinted from Clinical Immunology, Vol 109, Stolina M et al, Regulatory effects of osteoprotegerin on cellular and humoral immune responses, pp 347–354, © 2003, with permission from Elsevier.

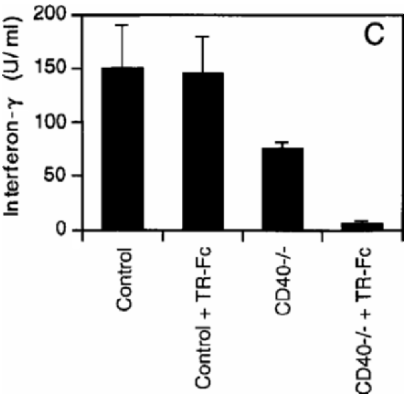

Figure 2. The effect of RANKL inhibition on virally-induced $CD4^+$ T cell responses and interferon γ production. C57BL/6 (control) mice or CD40-deficient (CD40−/−) mice were injected with LCMV and treated with TR-Fc (RANK-Fc) or hIgG1. Spleen cells were isolated 13 days after infection, and $CD4^+$ T Cells were purified and stimulated in vitro with LCMV-infected splenic APCs. Secretion of IFNγ was measured in culture supernatants by ELISA. Results shown are means ± SEM (n=3 mice per group). Reproduced with permission from the Journal of Experimental Medicine, 189: 1025–1031. Copyright 1999 The Rockefeller University Press.

was able to provide some increase in IFNγ production in response to an LCMV viral challenge, but only to a limited extent (Figure 2, bars 3 and 4). In the reverse situation (Figure 2, bars 1 and 2), when CD40 was present and the RANKL/RANK pathway was inhibited, no decrease was seen in the IFNγ response (Bachmann, Wong, Josien, et al. 1999).

Rare genetic deficiencies in CD40 or CD40L (also known as CD154) have been identified that are associated with severe combined immunodeficiency from birth. These patients are readily identified in infancy or early childhood because of the recurrent infections consequent to the loss of the CD40 pathway (Lougaris, Badolato, Ferrari, et al. 2005) and would be unlikely to be placed on a RANKL inhibitor inadvertently. When CD40 is present as usual, its stimulation is one of the most powerful activators in the immune system. In addition, CD40 upregulates OPG production in B cells and dendritic cells (Yun, Chaudhary, Shu, et al. 1998) and thus, activation of CD40 may lead to inhibition of the RANKL pathway as a normal physiologic consequence.

5. RANKL INHIBITION IN ANIMAL MODELS OF INFLAMMATION

Local and systemic bone loss are common in animal models of inflammatory disease (Byrne, Morony, Warmington, et al. 2005). In these models, RANKL inhibition clearly reduces bone loss but does not show a detectable effect on immune mediators or inflammation. For example, in a mouse model of inflammatory bowel disease driven by activated T cells ($CD45^+Rb^{hi}$ phenotype), RANKL inhibition had prominent effects on the preservation of bone mass; however, no detectable effects were seen on the immunologic infiltrates or inflammatory responses in the gut (Byrne, Morony, Warmington, et al. 2005).

Similarly, in rats or mice with inflammatory arthritis, bone and joint preservation was observed with RANKL inhibition, but no changes in joint swelling or in immune mediators were evident, regardless of which component of the immune system (B cell, T cell, cytokine)

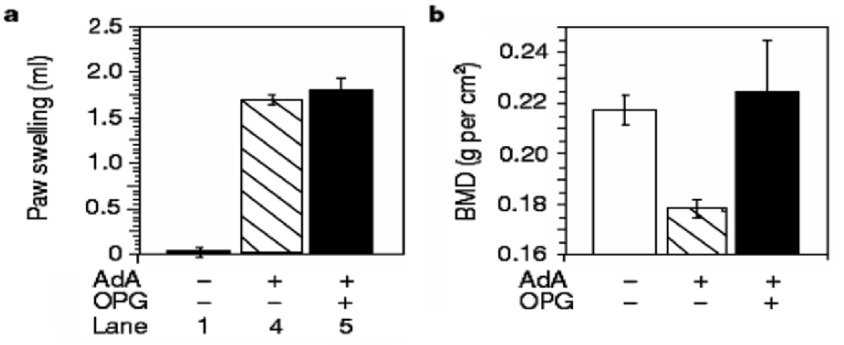

Figure 3. OPG prevents bone loss in adjuvant induced arthritis (AdA) but has no effect on paw swelling. a. Volume of hind paw swelling was calculated on day 16 after initiation of disease. Values shown are mean values of swelling (water displacement (ml)) ± SD, (n=6 per group). b. Bone mineral density (BMD) of the tibiotarsal region was determined on day 16. Values shown are mean values ± SD (n=6 per group). Adapted by permission from Macmillan Publishers Ltd: [Nature] (Kong et al. 1999, Nature 402:304-309), copyright © 1999.

was most predominant as the etiology of the joint disease (Kong, Feige, Sarosi, et al. 1999; Redlich, Hayer, Maier, et al. 2002; Romas, Sims, Hards, et al. 2002). In arthritic joints, RANKL is generated by activated synovial fibroblasts and lymphocytes and can be produced at very high levels (Kong, Feige, Sarosi, et al. 1999). RANKL recruits and activates osteoclasts and leads to the focal bone erosions at the joints and to the systemic bone loss that are characteristic of inflammatory arthritis. RANKL inhibition with OPG has shown a striking benefit on periarticular bone destruction and systemic bone loss (Figure 3), but despite the presence of RANKL on the lymphocytes, no effect on the inflammatory response in arthritic joints was seen (Kong, Feige, Sarosi, et al. 1999).

In summary, these results suggest that the RANKL/RANK pathway does not have an essential role in the adult immune system, in contrast to its essential role in mediating bone resorption.

6. RANKL INHIBITION WITH DENOSUMAB IN HUMANS

Denosumab (formerly AMG 162) is an investigational, fully human monoclonal antibody that binds to and inhibits RANKL. Like osteoprotegerin (OPG), denosumab inhibits bone loss by preventing RANKL from interacting with its receptor, RANK. In a randomized, placebo-controlled phase 2, dose-ranging study, 412 postmenopausal women with low bone mineral density (BMD) were treated with placebo, denosumab, or alendronate (McClung, Lewiecki, Cohen, et al. 2006). Denosumab treatment for 12 months increased lumbar spine BMD by 3.0% to 6.7%, compared with a change of −0.8% with placebo (P<0.001). Denosumab treatment also significantly increased BMD at the total hip, femoral neck, distal 1/3 radius, and total body compared with placebo. As seen in Figure. 4, at some sites, increases in BMD with denosumab were similar to increases observed with open-label alendronate. However, with the 60-mg, 6-monthly dose (the dose selected for phase 3 trials) denosumab treatment appeared to have greater effects than alendronate on BMD of two highly cortical sites, the total hip and distal 1/3 radius.

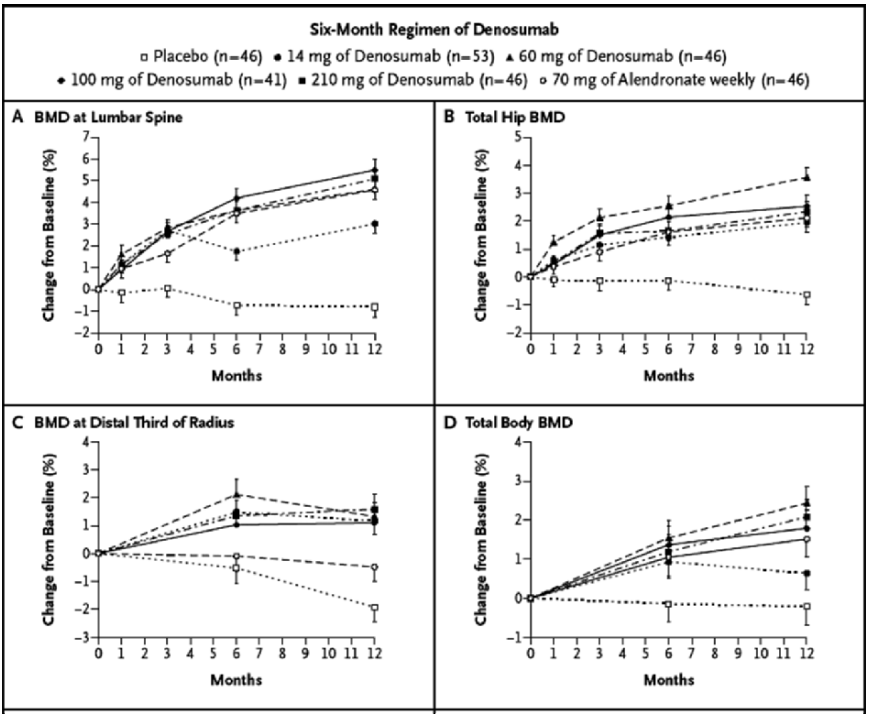

Figure 4. Changes in bone mineral density over 12 months of treatment with placebo, denosumab, or open-label alendronate. Mean percent changes from baseline in BMD at the lumbar spine (A), total hip (B), distal 1/3 radius (C), and total body (D) are shown for placebo, denosumab dosed every 6 months, or open label oral alendronate. McClung, Lewiecki, et al. 2006. N Engl J Med 354:821–831. Copyright © 2006 Massachusetts Medical Society. All rights reserved. Reprinted with permission.

Compared with placebo, reductions in the bone turnover markers serum C-telopeptide (CTX) and urine N-telopeptide (NTX) with denosumab were rapid (observed within 72 hours at the first time point measured), sustained, and reversible. At lower doses of denosumab, a release in the suppression of serum CTX levels was observed at the end of the dosing interval.

At 12 months the subject incidence of adverse events in the denosumab group was similar to the subject incidence of adverse events in the placebo- and alendronate-treated groups (87.3% denosumab, 89.1% placebo, 91.3% alendronate) (McClung, Lewiecki, Cohen, et al. 2006). After 2 years of treatment with denosumab the adverse event profiles continued to be similar among the three treatment groups and no new pattern of events occurred in the second year (Lewiecki, Miller, McClung, et al. 2005). The adverse events that occurred with greatest frequency in any dose group were arthralgia (19% denosumab, 28% placebo, 11% alendronate), dyspepsia (11% denosumab, 7% placebo, 26% alendronate); upper respiratory tract infection (24% denosumab, 17% placebo, 24% alendronate), and nausea (11% denosumab, 4% placebo, 22% alendronate). After 2 years the number of malignancies was balanced across treatment groups: 4.3% in the placebo-treated subjects, 2.2% in the open-label alendronate-treated subjects, and 3.2% in the denosumab-treated subjects.

Immune parameters were also examined in a phase 1 study of denosumab in healthy postmenopausal women (Bekker, Holloway, Rasmussen, et al. 2004). Denosumab doses of 0.01 to

3 mg/kg were evaluated. Patients received a single subcutaneous injection of denosumab or placebo and were monitored for up to 9 months. No changes in response to denosumab treatment were observed for white blood cell count, overall lymphocyte count, T-cell, B-cell, or NK-cell counts, or in immunoglobulin levels (IgM, IgG, IgA). Denosumab rapidly and significantly reduced urine and serum NTX levels compared with placebo in this study.

7. CONCLUSION

In conclusion, preclinical and clinical evidence suggest that inhibition of the RANKL pathway is different from complete absence of RANK or RANKL. Adult animals exposed to immune challenge in the presence of sustained RANKL inhibition mounted normal immune responses. In animal models of inflammatory disease with accompanying bone loss, RANKL inhibition inhibits bone loss but has no detectable effect on inflammation. Finally, in human clinical studies to date, RANKL inhibition with denosumab has been safe and well tolerated, inhibits bone resorption, and increases BMD.

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