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MEETING REPORTS

Osteoimmunology in the Aegean Sea. Meeting Report from the 2nd International Conference on Osteoimmunology: Interactions of the Immune and Skeletal Systems

June 8-13, 2008 in Rhodes, Greece

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Increasing evidence demonstrates that there are multiple levels of interaction between bone and immune cells. To better understand these and to develop new collaborations in this field, the 2nd International Conference on Osteoimmunology: Interactions of the Immune and Skeletal Systems was held in Rhodes, Greece from June 8-13, 2008 (http://www.aegeanconferences.org/).

Traditionally, bone cells like osteoblasts, osteoclasts and osteocytes or immune cells like lymphocytes, macrophages and granulocytes have been studied by scientists who are highly focused on selected areas of their function. However, to fully appreciate the mechanisms that regulate these cells, it is also necessary to understand their interactions. It has been more than 30 years since it was shown that cytokines can influence the function of osteoblasts and osteoclasts. However, the question of what roles these interactions have in the physiologic and pathologic function of bone cells remains unanswered. An explosion of studies examining these interactions occurred after the discovery of receptor activator of NF-κB ligand (RANKL) in 1998. This protein was first identified as a T cell-derived immunomodulatory molecule and then, shortly thereafter, was found to be the master regulator of the differentiation of osteoclasts from bone marrow precursors. The discovery of RANKL and its receptors (RANK and OPG) has helped to define the role of many different cytokines in bone metabolism. An ever-increasing number of pathways and transcription factors that were once thought to govern the immune system exclusively are now known to be utilized by bone cells. Furthermore, recent advances in defining the niche for hematopoietic stem cells (HSCs) in adult bone have assigned novel roles for osteoblasts in the development of the niche.

The 2nd International Conference on Osteoimmunology: Interactions of the Immune and Skeletal Systems featured three major topics: osteoblast lineage cells and their interaction with HSCs; the regulation of bone lineage cell differentiation; and the interactions of bone cells with inflammatory cells.

Niche and Soil

This session focused on the role that bone plays as an environment for the maintenance and development of bone

marrow cells and why certain tumors preferentially metastasize to bone.

Henry Kronenberg (Harvard Medical School, USA) presented data, from studies of chimeric mice that had a deletion of the guanine nucleotide binding protein stimulatory α subunit ($G_s\alpha$) (1). These studies used gene-deleted cells that also expressed a gene reporter (LacZ), which allowed their location in tissues to be monitored by enzyme histochemistry. The data demonstrated that hematopoiesis proceeded normally in the fetal liver of mice but the migration and engraftment of $G_s\alpha$-deficient HSCs from the fetal liver to the bone marrow was blocked. Surprisingly, lack of $G_s\alpha$ did not alter the ability of HSCs to mature *in vitro* or to migrate in response to stromal cell-derived factor-1 (SDF-1 or CXCL12). Conversely, pharmacologic stimulation of the $G_s\alpha$ pathway enhanced HSC homing and engraftment *in vivo*. These data demonstrate that a $G_s\alpha$-mediated pathway is involved in the ability of HSCs to migrate from the fetal liver and engraft in the bone marrow, which is an essential step in the development of the adult hematopoietic system. At this time, the nature of the ligand-receptor system(s) that requires $G_s\alpha$ to facilitate this process is unknown.

Theresa Guise (University of Virginia, USA) presented data on the role of transforming growth factor-β (TGF-β) in the ability of metastatic tumor cells to migrate to bone (2). TGF-β is produced and deposited in bone by osteoblasts and then released as an active form by osteoclastic resorption. Inhibition of TGF-β signaling with pharmacologic agents in *in vivo* murine models was found to inhibit the ability of metastatic tumor cells in bone to produce osteolytic metastases. This was true for multiple types of cancers, including breast cancer, prostate cancer and melanomas. However, inhibition of TGF-β receptor 1 kinase activity, which blocked TGF-β signaling in a murine *in vivo* model, increased the growth in bone of a prostate cancer cell line, which predominately produced increases in bone mass. These latter effects appeared due to the ability of the tumor to enhance osteoblast differentiation. Hence, inhibition of TGF-β signaling may have both beneficial and detrimental effects on bone, which will likely make it difficult to develop inhibitors of this pathway that can be used to treat metastatic prostate cancer in bone.

Basic Concepts in Osteoimmunology: Inflammatory Bone Diseases

This session focused on the role of synovial inflammation in the pathogenesis of deregulated peri-articular bone remodeling and the relationships between immune cells and osteoclast precursor cells.

Georg Schett (University Erlangen-Nuremberg, Germany) discussed the mechanisms of inflammatory bone destruction in rheumatic diseases such as rheumatoid arthritis (RA), ankylosing spondylitis (AS) and psoriatic arthritis and summarized recent data from animal models and human joint tissues. He showed that osteoclasts are the cell type responsible for focal bone resorption and discussed the role of locally produced cytokines, including RANKL as well as Th1 and Th17 cell-derived products in the induction of osteoclast differentiation in inflammatory tissue. Furthermore, an absence of T-regulatory (Treg) cells at inflammatory sites appeared to facilitate bone resorptive processes (3). Recent studies from Dr. Schett's group indicate that the defective bone repair that characterizes RA is in part related to TNF-induced production of the Wnt-pathway inhibitor DKK-1. In AS, which is characterized by localized increases in bone formation, this inhibitor may be absent or produced at low levels.

Debra Zack (Amgen, USA) reviewed the results from a completed phase 2, randomized, double-blind, placebo-controlled clinical trial designed to examine if a fully human monoclonal antibody to RANKL (denosumab) could preserve skeletal integrity in adult patients with active, erosive RA who were receiving maintenance methotrexate (4). Results indicated that denosumab produced a significant reduction in joint erosions and systemic bone loss. There were no significant adverse events associated with denosumab treatment in this study.

Edward Schwarz (University of Rochester, USA) proposed that TNF produced in joints (and additional sites) in RA stimulates the proliferation and release of CD11b+ myeloid precursors from the bone marrow. These cells enter the circulation and home to inflamed joints where they differentiate into osteoclasts, which are responsible for focal bone resorption. In studies employing TNF-transgenic (TNF-Tg) animals with inflammatory arthritis, Dr. Schwarz's group localized the production of osteoclast precursors to regions of "bone marrow edema", which were detected using a magnetic resonance imaging device that his group had developed. Microarray analysis of bone marrow from these sites suggested that the increased production and release of the CD11b+ cells was associated with increased production of VEGF-C and reduced levels of SDF-1. A striking increase in lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1)-expressing lymphatic vessels in the TNF-Tg mice and RA synovium was also detected. In TNF-Tg mice, these changes were associated with a 5-fold increase in the size of draining popliteal lymph nodes, supporting the speculations that there was a prominent role for altered lymphatic trafficking in inflammatory arthritis.

Yen-Tung Andy Teng (University of Rochester, USA) provided evidence that leukocytes with many characteristics of immature myeloid dendritic cells (DCs) can serve as osteoclast precursors, in addition to their ability to present antigen and regulate T cell function (5). The work from his lab showed that microbial or protein antigens together with T cells could induce osteoclast formation from immature DCs and that these induced osteoclasts (DDOC) could function as resorbing cells in models of inflammatory arthritis. Additional studies implicated TGF-β signaling as a critical factor regulating the early stages of DDOC formation.

Basic Concepts in Osteoimmunology: Osteoblasts

In this session, different aspects of the physiology of osteoblasts were presented. Emphasis was on pathways that are common to both immune cell development and osteoblast development.

Rudolf Grosschedl (Max Planck Institute, Germany) extended his observations of the effects that the early B cell factor (Ebf) family of transcription factors have on the interactions of osteoblasts with other cell types (6). Four members of the Ebf family have been identified (Ebf1-Ebf4). Ebf1 functions at the earliest stage of B cell differentiation, being required for commitment of progenitor cells to the B cell lineage. Ebf2, like Ebf1, is expressed in non-hematopoietic osteoblasts and adipocytes in bone marrow. Ebf2-deficient mice were used to examine the role that Ebf2 plays in regulating the stromal compartment of bone marrow. Adult, but not fetal, lymphopoiesis was markedly impaired in *Ebf2(-/-)* mice. Ebf2-expressing osteoblasts were observed on bone surfaces and in close association with hematopoietic progenitors. Gene expression profiling showed that genes associated with HSC differentiation (*i.e.*, *Notch1* and *Axin2*) were decreased in *Ebf2*(-/-) osteoblasts compared to controls. These data suggest that Ebf2 influences the ability of osteoblasts to maintain HSCs.

Dallas Jones and Laurie Glimcher (Harvard Medical School, USA), using compound mutant mice, showed the effects that the Schnurri family (Shn1, Shn2, and Shn3) of proteins have on osteoblast activity. The large zinc-finger Schnurri proteins have been shown to regulate numerous physiological processes. It was previously found that Shn3 is a key regulator of postnatal bone formation (7). To further elucidate the mechanism underlying the osteosclerotic phenotype of Shn3-deficient mice, they examined whether Shn3 regulates additional aspects of skeletal biology in parallel with the other mammalian Schnurri proteins. Towards this aim, mice deficient in both Shn2 and Shn3 were created. Skeletal formation in these mice was then compared to wild type mice and mice bearing single null mutations in Shn2 or Shn3. Analysis of compound Schnurri-deficient mice revealed an earlier onset and a more pronounced high bone mass phenotype than what is observed in Shn3 single mutant mice. These data suggest

multiple and overlapping roles for the Schnurri proteins in bone formation.

Mesenchymal precursor cells can be differentiated into adipocytes and osteoblasts *in vitro*. In addition, rates of adipogenesis in marrow are often reciprocal with rates of osteoblastogenesis in bone. Ormond MacDougald (University of Michigan, USA) reviewed this complex relationship in the context of Wnt signaling (8). Wnts comprise a family of secreted signaling proteins that regulate diverse developmental processes. Activation of Wnt signaling by Wnt10b inhibits differentiation of preadipocytes and blocks adipose tissue development. However, the effects of Wnt10b on mesenchymal lineages other than the adipocyte have not been well-defined. Transgenic mice that selectively express Wnt10b in adipocytes and macrophages (FABP4-Wnt10b mice) have previously been generated. To explore the physiological role of Wnt10b signaling in osteoblastogenesis and bone development, the bone phenotype of FABP4-Wnt10b mice was determined. Femurs from FABP4-Wnt10b mice had almost four times as much bone in the distal metaphysis, and were mechanically stronger. These mice maintain elevated bone mass at least through 23 months of age. In addition, FABP4-Wnt10b mice were protected from the bone loss of estrogen deficiency. Pharmacological and genetic approaches were used to demonstrate that canonical Wnt signaling stimulates osteoblastogenesis and inhibits adipogenesis in bipotential mesenchymal precursors. It was also found that Wnt10b shifts cell fate towards the osteoblast lineage by inducing the osteoblastogenic transcription factors, Runx2, Dlx5, and osterix, and suppressing the adipogenic transcription factors, C/EBPα and PPARγ. The rapid repression of adipogenic transcription factors appears to be necessary and sufficient for the stimulation of osteoblastogenesis by Wnt10b.

Toshihisa Komori (Nagasaki University, Japan) updated the mechanism by which osteoblast differentiation is regulated by Runx2 (9). Runx2 protein is first detected in preosteoblasts, and its expression is upregulated in immature osteoblasts and downregulated in mature osteoblasts. Runx2 is the earliest transcription factor required for the differentiation of precursors towards the osteoblast lineage, while Sp7 and canonical Wnt signaling further direct the fate of mesenchymal cells towards osteoblasts and away from chondrocytes. Runx2 induces the differentiation of multipotential mesenchymal cells into immature osteoblasts, directing the formation of immature bone, but Runx2 inhibits further osteoblast and bone maturation. Normally, the protein level of Runx2 in osteoblasts is reduced during bone development, when osteoblasts acquire a mature phenotype. Dr. Komori also showed that Runx2 triggers the expression of the major bone matrix genes during the early stage of osteoblast differentiation, but Runx2 is not essential for the maintenance of these gene expressions in mature osteoblasts.

Melissa Kacena (Indiana University, USA) provided evidence that megakaryocytes can stimulate osteoblasts and inhibit osteoclasts (10). Mice deficient in either the transcription factor GATA-1 or NF-E2, which are required for the terminal differentiation of megakaryocytes, exhibited a striking increase in bone mass. *In vitro* data suggest that megakaryocytes have pleiotropic effects on osteoclasts and osteoblasts. They express RANKL, suggesting that they may directly increase osteoclast formation. However, many investigators have shown that megakaryocytes also express and/or secrete factors that inhibit osteoclast development including: osteoprotegerin, IL-10, IL-13, TGF-β, and GM-CSF. Megakaryocytes and megakaryocyte-conditioned medium significantly inhibit *in vitro* osteoclastogenesis. With regard to modulating osteoblast function, it was found that megakaryocytes express and/or secrete several bone-related proteins, including osteocalcin, osteonectin, bone sialoprotein, osteopontin, BMP and osteoprotegerin. These proteins play a role in bone formation and remodeling. Recent studies have demonstrated that megakaryocytes can directly stimulate osteoblast proliferation and have a dramatic effect on osteoblast differentiation *in vitro*. Specifically, co-cultures of megakaryocytes and osteoblasts resulted in an up to six-fold increase in osteoblast proliferation by a mechanism that

required direct cell-to-cell contact. The heightened proliferative response is mirrored by a reciprocal decrease in osteoblast differentiation as measured by gene expression, alkaline phosphatase activity and mineral deposition. Overall, these findings illustrate that there are complex regulatory interactions between megakaryocytes and bone cells.

Basic Concepts in Osteoimmunology: Osteoclasts

This session focused mainly on the relationships between osteoclasts and the immune system. The speakers described how osteopenia and periarticular bony erosion result from chronic inflammatory autoimmune disease because of an imbalance between osteoclast activity and new bone formation.

Mary Nakamura (University of California, San Francisco, USA) and Lionel Ivashkiv (Hospital for Special Surgery, USA) described how costimulatory receptors such as TREM2 (triggering receptor expressed in myeloid cells-2), which associates with the signaling adapter DAP12, regulates osteoclast function independently from its effects on osteoclast differentiation.

Steven Teitelbaum (Washington University, USA) presented data demonstrating that synaptotagmin VII, a calcium-sensing protein that regulates exocytosis, is associated with lysosomes in osteoclasts and bone matrix protein-containing vesicles in osteoblasts (11). The absence of synaptotagmin VII inhibited cathepsin K secretion and the formation of a ruffled border in osteoclasts and bone matrix protein deposition by osteoblasts. Reflecting a dominance of inhibited osteoblast function relative to that of osteoclasts, synaptotagmin VII-deficient mice are osteoporotic

Hiroshi Takayanagi (Tokyo Medical and Dental University, Japan) presented an extensive overview of how multiple transcription factors are shared among osteoclast lineage cells and immunocytes. Anthony Aliprantis and Laurie Glimcher (Harvard Medical School, USA) described their analyses of newly generated NFATc1 conditional knockout mice, and the role that NFATc1 has in osteoclast differentiation.

Sakae Tanaka (University of Tokyo, Japan) presented *in vivo* and *in vitro* studies, which described how osteoclast apoptosis is regulated by Bcl-2 family members. Osteoclasts generated from Bcl-xL-deficient mice exhibited a shorter life span and higher bone-resorbing activity than did osteoclasts generated from normal littermates. These results suggest an important role for Bcl-xL in regulating both the apoptosis and activation of osteoclasts.

Up-and-Coming

The speakers at the final session of the meeting discussed novel aspects of osteoimmunology.

Josef Penninger (Institute of Molecular Biotechnology, Austria) described the role of the RANKL-RANK system outside of bone. RANKL can be induced by UV irradiation in skin keratinocytes and this signal is proposed to rewire Langerhans cells in the skin to promote proliferation of regulatory T cells. Transgenic overexpression of RANKL in keratinocytes results in an increased number of Treg cells and suppression of local hypersensitivity responses in the skin, as well as systemic CD40L-driven autoimmunity. Interestingly, vitamin D, which is also produced in the skin in response to sun exposure, has long been known to have immunosuppressive functions and to induce RANKL in bone. Thus, RANKL-RANK might be the missing link mediating sunlight-induced immunosuppression. Dr. Penninger also discussed surprising recent data that RANKL is a key cytokine that triggers the development of autoimmune regulator (AIRE)-expressing medullary thymic epithelial cells (mTECs). Thus, RANKL-RANK appears to have an additional function in central tolerance in the thymus via AIRE+ mTECs. Dr. Penninger also presented a new mouse model with a floxed allele of RANK, which permits tissue specific knockout of this gene. Considering the complex phenotypes of total RANKL or RANK mutant mice, which range from severe osteopetrosis, defective mammary gland formation in pregnancy, defective

lymph node organogenesis, or impaired development of AIRE+ mTECs, such mice will be valuable for dissecting the functions of RANKL-RANK signaling in various tissues.

The acute phase response is the major adverse effect of intravenously administered nitrogen-containing bisphosphonates (N-BPs), which are frequently used to treat a variety of metabolic bone diseases. Keith Thompson (University of Aberdeen, UK) presented a very intriguing and convincing model to explain why N-BPs such as zoledronic acid trigger an acute phase immune response. Following a typical intravenous N-BP infusion, these drugs are internalized by highly endocytic cells in peripheral blood such as monocytes. Once internalized, N-BPs potently inhibit the enzyme farnesyl diphosphate (FPP) synthase, resulting in the intracellular accumulation of the substrates of FPP synthase, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) selectively in monocytes. Since IPP and DMAPP are agonists for the T cell receptor expressed on γδ T cells, N-BPs indirectly activate γδ T cells by triggering the intracellular accumulation of IPP and DMAPP in monocytes, which is then presented to the γδ T cell. Activation of γδ T cells results in the production of pro-inflammatory cytokines such as TNFα, IFNγ and IL-6, which are thought to drive the flu-like symptoms characteristic of this phenomenon. In addition, Dr. Thompson also demonstrated that statins, by inhibiting an upstream enzyme in the same biosynthetic pathway targeted by N-BPs, prevent N-BP-induced IPP and DMAPP accumulation, and thereby prevent N-BP-induced γδ T cell activation *in vitro*. This raises the possibility that statins may provide a pharmacological means for preventing the acute-phase response to N-BPs in patients.

Naoyuki Takahashi (Matsumoto Dental University, Japan) presented data that osteoblasts define the sites of osteoclast development. Injection of RANKL into RANKL-deficient mice only triggers osteoclast formation in bones. Dr. Takahashi also discussed a new concept that cell cycle-arrested osteoclasts maintain the pre-osteoclast niche, which allows cells to develop into osteoclasts independent of cell cycle progression. He has termed this potential novel progenitor QOP (quiescent osteoclast precursor) and determined that it can be found in the bone marrow as well as the peripheral blood.

T. Jack Martin (St. Vincent's Institute of Medical Research, Australia) discussed the role of ephrins and Eph receptors in osteoblasts. Transgenic overexpression of EphB4 in osteoblasts under the control of a Col1a promoter increased osteoblast numbers and decreased bone resorption. EphB4 made by osteoblasts produces responses by binding to ephrin B2, which is expressed on both osteoblasts and osteoclasts. Dr. Martin presented evidence that PTH induces EphrinB2 production by osteoblasts, which may be one mechanism by which PTH produces an anabolic effect in bone (12). Action of EphrinB2 within the osteoblast lineage was shown by using blockers of EphrinB2-EphB4 receptor interaction, which decreased osteoblast mineralization and the expression of several osteoblastic genes.

Summary

It became clear at this meeting that osteoimmunology is a broad field. Interactions of immune and bone cells occur at multiple levels including the development of the bone marrow microenvironment, the regulation of bone turnover in health and disease and the development of osteoclasts from a common myeloid precursor, which also gives rise to dendritic cells and macrophages. Finally, new pathways regulating both bone and immune cells have recently been discovered, and pathways that were originally identified as critical for bone and immune cell interactions have now been found to have effects on multiple organ systems.

Conflict of Interest: Dr. Goldring reports receiving a research grant from Boehringer Ingelheim. Dr. Lorenzo and Dr. Penninger report owning stock in Amgen. Dr. Choi, Dr. Horowitz, Dr. Takayanagi and Dr. Zallone: none reported.

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