

ACTIVITY OF THE LABORATORY FOR THE STUDY OF SKELETAL DISORDERS AND REHABILITATION

GLIMCHER MJ, HAUSCHKA PV, HOFSTAETTER JG, MCHUGH KP, SALIH E, SAMUEL RE, SOLOMON KR, WANG J, WU Y, WUNDERLICH L

LABORATORY FOR THE STUDY OF SKELETAL DISORDERS AND REHABILITATION, DEPARTMENT OF ORTHOPAEDIC SURGERY, CHILDREN'S HOSPITAL, HARVARD MEDICAL SCHOOL, BOSTON MA

The following is a summary of ongoing research projects in the laboratory for the study of skeletal disorders and rehabilitation at the Children's Hospital. Names listed below the titles are those of the principal investigators working on the various projects.

THE ROLE OF OXYGEN IN SKELETAL DEVELOPMENT

Raymond E. Samuel, M.D., Ph.D.

The molecular characterization of the highly ordered differentiation of epiphyseal growth plate chondrocytes has advanced significantly over the last decade. A new group of autocrine/paracrine factors (parathyroid hormone related-protein, PTHrP; Indian Hedgehog, Ihh; fibroblast growth factor, FGF) and their associated receptors (PTHrP-receptor; Patched, Ptc; FGF receptor 3, FGFR3) have been added to the well-established endocrine regulators of longitudinal skeletal growth. Transgenic "knock-out" mice and viral over-expression in avian systems have provided key insights into the feedback loop in which the Ihh factor, produced by the prehypertrophic chondrocytes of the growth plate, binds to Ptc receptors on the surface of cells of the perichondrium. These perichondrial cells

then up-regulate the production of PTHrP, which binds to the PTHrP-receptors located on the surface of the prehypertrophic chondrocytes. The signal transduction resultant from the PTHrP/PTHrP-receptor complex leads to a down-regulation of Ihh production by the prehypertrophic chondrocytes and the regulation of their rate of differentiation to the terminally differentiated hypertrophic chondrocytes.

Additionally, oxygen delivery to the growth plate (i.e. vascular invasion) is critical for the transformation of the hypertrophic chondrocyte to the metaphyseal bone of the primary spongiosa. The lack of angiogenic stimulus in hypertrophic chondrocytes (conditional knock-out of VEGF isoform and Cbfa1 knock-out) results in delayed progression through the process of endochondral ossification. Hence, systemic changes in oxygen delivery to the growth plates of long bones, as seen clinically in hypoxic diseases (congenital cyanotic heart disease, asthma, sickle cell disease) and following systemic therapy with novel anti-angiogenic medications (Endostatin, Entremed Inc., Rockville, MD), may likewise result in modulation in growth plate chondrocyte differentiation dynamics and consequently alterations in the rate of longitudinal long bone growth.

My research is directed toward the understanding of the mechanism by which alterations in the oxygen supply to the epiphyseal growth plate chondrocytes of long bones influence the process of endochondral ossification. Therein, I utilize molecular (gene transfer via non-viral and viral vectors) and environmental (systemic hypoxia induced via housing animals in an hypoxic chamber) techniques towards limiting the supply of oxygen available to the growing embryonic and post-natal long bones, respectively. Initial results of the chronic hypoxia model strongly suggest a direct influence of oxygen concentration on the regulatory pathway(s) of endochondral ossification. Moreover, the decreased oxygen concentration additionally affected the rate and/or extent of both metaphyseal cancellous and cortical bone mineralization of the growing long bones.

Dr. Glimcher is the Harriet M. Peabody Professor of Orthopaedic Surgery, Harvard Medical School and Director of the Laboratory for the Study of Skeletal Disorders and Rehabilitation, Department of Orthopaedic Surgery, Children's Hospital, Boston, MA.

Dr. Hauschka is Associate Professor of Oral and Developmental Biology, Harvard School of Dental Medicine and Research Associate in the Laboratory for the Study of Skeletal Disorders and Rehabilitation, Department of Orthopaedic Surgery, Children's Hospital, Boston, MA.

Dr. Salih is an Assistant Professor of Orthopaedic Surgery, Harvard Medical School and Research Associate in the Laboratory for the Study of Skeletal Disorders and Rehabilitation, Department of Orthopaedic Surgery, Children's Hospital, Boston, MA.

Dr. Solomon is an Assistant Professor of Orthopaedic Surgery, Harvard Medical School and Research Associate in the Laboratory for the Study of Skeletal Disorders and Rehabilitation, Department of Orthopaedic Surgery, Children's Hospital, Boston, MA.

Dr. McHugh, Wang and Wu are Instructors in Orthopaedic Surgery, Harvard Medical School and Research Associate in the Laboratory for the Study of Skeletal Disorders and Rehabilitation, Department of Orthopaedic Surgery, Children's Hospital, Boston, MA.

Dr. Hofstaetter, Samuel and Wunderlich are Research Fellows in Orthopaedic Surgery, Harvard Medical School and the Laboratory for the Study of Skeletal Disorders and Rehabilitation, Department of Orthopaedic Surgery, Children's Hospital, Boston, MA.

Please address correspondence to:

Dr. Melvin J. Glimcher
Laboratory for the Study of Skeletal Disorders and Rehabilitation
Children's Hospital
300 Longwood Avenue
Boston, MA 02115

BONE CELL BIOLOGY GROUP

Peter V. Hauschka, Ph.D. and Keith R. Solomon, Ph.D.

Our main areas of current research include: 1) RANKL-ligand (Receptor Activator of Nuclear Factor-kappaB Ligand) RANK signaling in the osteolytic metastasis of breast cancer; 2) cholesterol and prostate cancer progression; 3) bone formation and vascular calcification by vascular pericytes; and 4) mechanical strain regulation of osteoblasts and osteoclasts.

Breast cancer metastases in bone pathologically stimulate osteoclasts, resulting in osteolytic bone destruction, fracture, nerve compression, and pain. Osteolysis provides fertile sites for tumor expansion, releasing growth factors stored in bone matrix and stimulating tumor proliferation and angiogenesis to nourish the metastatic foci. Breast cancer cells constitutively express RANK, making them potential targets for RANKL, with important consequences for metastasis in skeletal sites. Our general goal is to understand the mechanisms by which this

RANKL-RANK signaling pathway may promote the development of bone metastases and foster cell survival and tumor progression in metastatic breast cancer.

Pericytes are hypothesized to play a central role in the vascular calcification processes. Their capacity to differentiate into osteoblast-like cells in response to local molecular and biomechanical signals may explain the pathological calcification of atherosclerotic lesions. Pericytes are also a potential target for cholesterol-lowering “statin” drugs. Statins are believed to modulate the cholesterol-rich pre-assembled signaling complexes in cell membranes (caveolae and lipid rafts), and may also reduce the prenylation and lipid partitioning of accessory signaling proteins. Characterization of the cell-cell interactions and signaling pathways in pericytes that initiate and sustain their osteogenic transdifferentiation could provide the foundation for new therapeutic strategies in orthopaedic surgery, as well as allowing treatment of cardiovascular calcification and atherosclerosis.

References

1. **Dolan, M. M. and Hauschka, P. V.** Osteoprotegerin and RANK-Fc: novel therapies for pathologic osteolysis. *Orthopaedic Journal at Harvard Medical School* 2002; 4: 97-100.
2. **Nielsen-Marsh, C. M., Ostrom, P. H., Gandhi, H., Shapiro, B., Cooper, A., Hauschka, P. V., Collins, M. J.** Sequence preservation of osteocalcin protein and mitochondrial DNA in bison bones > 55ka. *Geology* (in press).

NFATp CONNECTION TO BONE HEALING

Livius Wunderlich, Ph.D.

NFATp is a member of the NFAT (Nuclear Factor of Activated T-cells) transcription factor family. Upon T cell activation, the NFATs dephosphorylate and translocate into the nucleus to activate genes involved in cell-differentiation. As soon as the NFATs rephosphorylate, they translocate back to the cytoplasm. NFATp-deficient mice have been previously generated. Mice lacking NFATp developed skeletal abnormalities and extra-skeletal masses of calcified cartilage. Absence of NFATp increased the expression of cartilage markers such as type II and type X collagen.

Our goal is to investigate the effects of the NFATp transcription factor on the expression of different proteins involved in bone production during fracture healing and cartilage production during articular cartilage repair. Candidate proteins were typical marker proteins, such as morphogenic proteins (BMPs), as well as bone and cartilage matrix proteins. In the future we plan to find the direct connection between the NFATp and the gene(s) for which NFATp stimulates expression.

Previously, we have drilled small holes into the tibia of wild type (WT) and NFATp-deficient mice. The healing tissue was harvested 3, 7, 10 and 14 days after the surgery. After total RNA isolation, the RNA level of collagen types I, II, X, and XII, osteocalcin, biglycan, decorin and aggrecan was investigated using real-time PCR. Investigation of other bone- and cartilage-related proteins is under consideration. This method will help us to detect real gene expression differences in NFATp versus WT mouse during the bone healing process.

One set of healing tibial tissue has already been investigated. According to the preliminary data, the following genes have increased mRNA levels in the healing tibiae of the NFATp knock-out mice: aggrecan and collagen type X on days 7 and 10, decorin on day 10 and collagen type II on day 3. We found decreased collagen type I mRNA level on day 3 in the NFATp-deficient animals. The experiment needs to be repeated to confirm these observations. However, these data suggest that NFATp has an inhibitory effect on the cartilage-related gene expression during the bone healing process. The lack of the NFATp leads to an endochondrial-like bone formation during the healing of the long bones.

EFFECT OF GLYCOSYLATION AND PHOSPHORYLATION ON BONE SIALO PROTEIN

Livius Wundelich, Ph.D.

Bone sialoprotein (BSP) is one of the important extracellular matrix proteins of bones and teeth. The *in vivo* phosphorylated, sulfated and highly glycosylated protein stimulates matrix mineralization. BSP is able to bind to osteoblasts and mediate cell attachment, thereby regulating osteoblast function.

Although the bovine BSP cDNA had been cloned in 1994, recombinant protein had never been used for *in vivo* experiments. Early *in vitro* studies showed that unsulfated BSP appears to be equivalent in its activity. The question of whether the phosphorylation and glycosylation have any effect on the function of the protein remains unsolved.

For *in vitro* and *in vivo* mineralization experiments, we decided to overexpress the full-length, mature bovine BSP protein. Proteins without secondary modifications have been produced in bacterial cells (*Escherichia coli*), while highly glycosylated and phosphorylated proteins have been overexpressed in yeast cells (*Pichia methanolica*). We are also interested in the difference in the biological effect of two fragments of the BSP protein: the serine and glutamate rich N-terminal domain with many possible phosphorylation sites, and the shorter C-terminal domain with less serines and glutamic acids.

Thusfar, we have constructed, overexpressed, and successfully purified the bovine BSP protein and its fragments in bacteria and yeast. After finishing the large-scale preparations, we will use the purified proteins for mineralization studies.

OSTEOCLASTIC DIFFERENTIATION

Kevin McHugh, Ph.D.

While required for normal skeletal growth and fracture healing, osteoclastic bone resorption is responsible for bone loss in osteoporosis, cancer metastasis to bone, and aseptic loosening of orthopaedic implants. Little is known, however, about regulation of the genes essential for the formation and function of osteoclasts.

It was recently established that osteoclasts differentiate from macrophage precursors through the interaction of Receptor Activator of NF- κ B Ligand (RANKL) with its receptor RANK. RANKL stimulated RANK, in turn, activates downstream signaling pathways including the transcription factors NF- κ B and Fos/Jun. The importance of these pathways in osteoclast formation and function is underscored by the fact that RANK and RANKL knockout mice, as well as the p50/p52 NF- κ B double knockouts, have no osteoclasts and are severely osteopetrotic. In addition, cfos knockout mice have osteoclasts that fail to polarize and therefore display an osteopetrotic phenotype. While critical for osteoclast formation, induction of the transcription factors NF- κ B and Fos/Jun is insufficient to explain gene expression, differentiation, and the function of osteoclasts.

We have adopted several strategies aimed at identifying transcriptional machinery in osteoclasts. These include: studies of osteoclast gene promoters, cDNA expression profiling, and direct identification of osteoclast nuclear DNA binding factors.

Our promoter studies target expression of the β 3 inte-

grin gene as a model of osteoclast gene expression. The β 3 gene is not expressed in osteoclast precursors. However, high levels of expression are required for normal osteoclast formation and function. In collaboration with Deborah L. Galson of the University of Pittsburgh, we find that cotransfection with NFATc1 enormously up-regulates (50X) β 3 integrin promoter-reporter activity. Dr. Galson has similar data for the calcitonin receptor, which is another osteoclast gene. NFATc1 was recently reported as an osteoclast transcription factor. Retroviral expression of NFATc1 was shown to induce osteoclastic differentiation without RANKL treatment. In addition, embryonic stem (ES) cells lacking NFATc1 are deficient in osteoclast formation. The β 3 integrin gene promoter contains two NFAT sites in the region from -401 nt to -370 nt relative to the transcriptional start site. Mutations of either or both of these sites will determine the sequences mediating NFAT induction.

Through our expression profiling experiments we have identified the transcription factor Stra13 as a RANKL induced factor in both human and mouse osteoclasts. Stra13 (a.k.a. DecI, SharpI) was first identified as a factor induced by retinoic acid in neuronal differentiation of mouse P19 embryonic carcinoma cells. Transgenic overexpression of Stra13 induces neuronal differentiation and gene expression in P19 cells in the absence of additional stimuli. In preadipocytes, Stra13 is a hypoxia inducible gene under the HIF-1 transcription factor and mediates hypoxic inhibition of adipogenesis. Stra13 is a member of the basic helix-loop-helix (bHLH) family of transcription factors and is most closely related to the Drosophila Hairy/Enhancer of Split (HES) transcription factors. Members of the

HES family are transcriptional repressors that generally inhibit cell division and direct cellular differentiation. Stra13 has been shown to act as a transcriptional repressor of basal and induced transcription. However, Stra13 reportedly does not display binding activity toward bHLH or HES consensus DNA binding sites. Stra13 likely represses transcription through interaction with the basal transcription machinery via TBP or TFIIB.

We find, by quantitative real-time RT-PCR, that primary macrophage and the preosteoclast cell line RAW264 express low levels of Stra13. Treatment of both cell types with RANKL induces Stra13 expression in a time dependent manner over the 5-6 day course of osteoclast differentiation. Western blots confirm Stra13 protein is expressed with osteoclast formation. We are currently overexpressing Stra13 and will determine its effects on osteoclast gene expression and differentiation.

USE OF ALENDRONATE TO DIMINISH SUBCHONDRAL BONE RESORPTION FOLLOWING OSTEONECROSIS OF THE FEMORAL HEAD IN RABBITS

Jochen G. Hofstaetter MD; Jinxi Wang MD, PhD; Melvin J. Glimcher MD

Osteonecrosis of the femoral head is a disease where death of osteocytes can lead to structural failure, collapse, and hip joint destruction. It is estimated to afflict approximately 15,000 new patients per year in the United States, with an average age of 36 years . After the initial ischemic event, a process of repair is initiated, where on the surfaces of the dead bony trabeculae new bone is formed. Three-dimensional (3-D) volumetric density of newly created bone is greatly increased. This leads to the increased density observed on plain radiographs. This newly formed bone is later resorbed by osteoclasts, which are of hematopoietic origin, and occurs during or following the revascularization of the necrotic tissue . It was found that necrotic bone retains load bearing capacity ; consequently the death of bone cells does not cause structural failure. Rather, structural failure is caused by the resorption of necrotic bone . If the bone resorption associated with osteonecrosis can be inhibited or delayed until sufficient new bone has formed, it would appear that structural failure and its consequences could be avoided.

Osteoclastic activity can be reduced with bisphosphonates, a class of drugs in clinical use for the treatment of osteoporosis, Paget's disease and osteolytic metastases. Bisphosphonates bind

to bone mineral and when resorbed by osteoclasts, bisphosphonates with cell metabolism, leading to apoptosis. This study is performed to investigate whether alendronate can inhibit or delay subchondral bone resorption until new bone has formed and to show that structural failure and its consequences can thus be avoided.

In our experimental model, osteonecrosis of the femoral is induced surgically in 100 young adult male New Zealand White rabbits. Fifty rabbits were used as untreated controls, while 50 rabbits were treated with Alendronate 3 times per week by subcutaneous injection. The rabbits were euthanized at times 1, 3, 6 and 12 months postoperatively. Tissue was harvested and analyzed by plain radiographs, micro-computed tomography (CT), and histology.

MicroCT allowed for the evaluation beyond simple two-dimensional orientations and radiographic densities. The microCT can accurately resolve micron-sized struts that make up cancellous bone. From these images a wide array of parameters that have been demonstrated to be related to bone mechanical properties can be measured. The high spatial resolution of 3D Micro-CT will make it possible to visualize and quantify the 3-D volumetric changes in the trabecular bone during the repair process and to visualize and quantify the resorption of the subchondral bone and compare the treated versus the untreated group.

PROTEIN KINASES

Erdah Salih, Ph.D.

Our laboratory has been involved in the study of protein kinases and delineation of the structure-function relationship of extracellular matrix (ECM) phosphoproteins of bone, dentin and enamel. In these studies a number of state-of-the-art (solid-phase N-terminal peptide sequence and matrix-assisted laser desorption/ionization-time of flight-mass spectrometry, MALDI-TOF-MS) and advanced protein chemistry approaches have been utilized for the first time in the field of orthopaedic and mineralized tissue studies.

As our understanding of the biological functions of bone ECM phosphoproteins advances, the significance of the state of phosphorylation is emerging. Conceptually, if the covalently bound phosphate groups are important in biological functions of osteopontin (OPN) and bone sialoprotein (BSP), then biochemical factors or processes that affect the state of phosphorylation of these proteins become biologically significant. *In vitro* studies using purified native and dephosphorylated OPN and BSP from bovine bone with a series of pure individual protein kinases indicated that the major and predominant kinase is the factor independent protein kinase, CKII¹. Other protein kinases phosphorylate OPN and BSP to a lesser extent. Other native OPN and BSP were found to be partially phosphorylated (88% and 65%, respectively).

The specific biological functions of individual phosphorylation regions and protein kinases are not presently known. However, they may play important and different functional roles ranging from mineral deposition to modulation of cellular activity and signal transduction at different stages of bone development, maturation and age. For instance, dephosphorylated OPN and BSP do not bind to osteoclasts², which led to the hypothesis that the highly phosphorylated N-terminal non-RGD domains of OPN and BSP strongly assist cell attachment¹. It was further postulated that the overall cell attachment properties of these proteins require more than a single functional domain or sequence, leading to the hypothesis that cell attachment/modulation involves participation of (a) the RGD sequence region, (b) phosphorylation regions, and (c) non-RGD amino acid sequence(s) in a coupled fashion¹. The functional consequences of coupling or synergistic effect of these different moieties clearly require systematic investigations. Studies using mineralizing cultured chicken osteoblasts led to the quantitative isolation of metabolically ³²P-labeled OPN and each phosphorylated peptide regions (including the precise site of phosphorylation within each peptide) were identified for the first time by automated N-terminal solid-phase amino-acid³. The sites of phosphorylation were predominantly

in peptides with amino acid recognition sequences [SXE(D)/SXSSEE(DD)/E(D)XSXX] for CKII. Of all of the phosphorylation sites (6 serines and 1 threonine) approximately 70% of the total phosphorylated residues were by CKII and the remaining 3 sites (30%) by other kinases. Overall, metabolic phosphorylation of OPN led to 10 phosphorylated residues (9 serines and 1 threonine), distributed almost equally on the two halves of the protein.

This series of original studies carried out in our laboratory^{1,3-5} formed the basis for novel concepts in both bone and other biological systems involving study of OPN and BSP, *e.g.* use of *in vitro* phosphorylation of recombinant OPN by commercial CKII in atherosclerosis, osteoclast binding/signaling⁶⁻⁷. Both the *in vivo* and *in vitro* phosphorylation sites of BSP were recently characterized and determined to be overlapping, predominantly at CKII recognition sequences, *e.g.* SSEE, SXEE⁸. Such studies were performed using a combination of protein sequence analysis, the latest MALDI-TOF-MS and novel reagents developed in our laboratory specifically to study phosphorylation phenomenon^{8,9}.

The phosphorylation state of OPN and BSP was determined in both a bony and a soft tissue environment. There was substantial variation in the rate of deposition of Ca⁺², OPN and BSP as a function of time in both implant sites, and significant differences in the quantities of these components between calvarial and subcutaneous bone formation^{10,11}. The levels of these components were approximately 5-fold lower throughout the implant period in soft tissue environment. These data indicate that the same inductive material (demineralized bone matrix, DBM) in two distinct environments induced different cellular and biochemical events. Whether such processes are the result of differences in the origin and nature of the local cells or the influence of the soft tissue environment on such progression to form bone is not easy to discern. In these models the rate of calcium deposition had a direct relationship with the ratio of phosphorylation state of BSP/OPN in the calvarial bony environment; such correlation was not observed in the soft tissue environment¹¹. This study highlighted the hidden facets of the process of finely controlled biomineralization.

In order to further define the domains of BSP/OPN responsible for the regulation of biomineralization/ bone remodeling, they are evaluated for their effect in neonatal mouse calvarial bone organ cultures. In a different set of studies we are using bone organ cultures in combination with proteomics/phosphoproteomics (MALDI-TOF-MS) to identify the factors that are released during bone remodeling. These studies promise to lead to the identification of yet unknown regulators of bone remodeling.

References

1. Salih, E., Wang, J., Mah, J., Fluckiger, R. Natural variation in the extent of phosphorylation of phosphoproteins as a function of *in vivo* new bone formation induced by demineralized bone matrix in soft tissue and bony environments. *Biochem J.* 2002;364:465-474.
2. Salih E, Zhou H-Y & Glimcher MJ. Phosphorylation of purified bovine bone sialoprotein and osteopontin by protein kinases. *J Biol Chem* 1996;271: 16897-16905.
3. Ek-Rylander, B, Flores, M, Wendel, M, Heinegard, D. & Andersson, G. Dephosphorylation of osteopontin and bone sialoprotein by osteoclastic tartrate-resistant acid phosphatase. Modulation of osteoclast adhesion *in vitro*. *J Biol Chem* 1994;269: 14853-14856.

4. **Salih E, Ashkar S, Gerstenfeld LC, Glimcher MJ.** Identification of the metabolically phosphorylated sites of secreted ³²P-labeled osteopontin from cultured chicken osteoblasts. Solid-phase N-terminal sequencing of phosphorylation sites of osteopontin. *J Biol Chem* 1997;272:13966-13973.
5. **Salih E, Ashkar S, Gerstenfeld LC, Glimcher MJ.** Protein kinases of cultured chicken osteoblasts: Selectivity for extracellular matrix proteins of bone and their catalytic competence for osteopontin. *J Bone Miner Res* 1996;11:1461-1473.
6. **Salih E, Ashkar S, Zhou H-Y, Gerstenfeld LC, Glimcher MJ.** Protein kinases of cultured chicken osteoblasts that phosphorylate extracellular bone proteins. *Connect Tissue Res* 1996;34(4)/35:(1-4):207-213.
7. **Jono, S, Peinado, C & Giachelli, CM.** Phosphorylation of osteopontin is required for inhibition of vascular smooth muscle cell calcification. *J Biol Chem* 2000;275: 20197-20203.
8. **Katayama, Y, House, CM, Udagawa, N, Kazama, JJ, McFarland, RJ, Martin, TJ, Findlay, DM.** Casein kinase 2 phosphorylation of recombinant rat osteopontin enhances adhesion of osteoclasts but not osteoblasts. *J Cell Physiol.* 1998;176(1):179-87.
9. **Salih, E.** In vivo and in vitro phosphorylation regions of bone sialoprotein. *Connect Tissue Res*, 44 Suppl. 2003;1 223-229.
10. **Salih, E.** Synthesis of a radioactive thiol reagent, 1-S-[³H]carboxymethyl-dithiethanol: identification of the phosphorylation sites by N-terminal sequencing and MALDI-TOF-Mass spectrometry. *Anal. Biochem*, in press.
11. **Wang, J, Glimcher, MJ, Mah, J, Zhou, H-Y, and Salih, E.** Expression of bone microsomal casein kinase II, bone sialoprotein, and osteopontin during the repair of calvarial defects. *Bone* 1998;22: 621-628.
12. **Salih, E, Wang, J, Mah, J, Fluckiger, R.** Natural variation in the extent of phosphorylation of bone phosphoproteins as a function of in vivo new bone formation induced by demineralized bone matrix in soft tissue and bony environments. *Biochem J.* 2002;364:465-474.

CHARACTERIZATION OF MORPHOLOGIC, CELLULAR AND MOLECULAR RESPONSE TO OSTEOGENIC MORPHOGENS IMPLANTED IN BONE DEFECTS AND IN SOFT TISSUE SITES

Jinxi Wang, M.D., Ph.D.

Understanding of cellular and molecular response to osteo-inductive materials in bone repair is of great significance to the clinical application of bone repair materials. We have carried out experiments to characterize the path of cell differentiation and gene and protein expression following the implantation of decalcified bone matrix (DBM) in cranial defects and soft tissue sites of rats.

The results have demonstrated that the implantation of DBM into cranial defects first induced the proliferation and differentiation of mesenchymal stem cells from the dura to alkaline phosphatase staining osteoblasts at approximately 3 days. These cells synthesized bone matrix that was calcified thereafter. Small clusters of cartilage cells with safranin-O staining matrix were first observed on days 6-7, then were spatially separated from the new bone and progressively resorbed and replaced with bone (1). To identify the origins of bone-forming cells, two specially designed experimental models were utilized to separate DBM implants from the host bone. Cells in the dura were labeled with ³H-thymidine and found to be the source of the osteoblasts, whereas undifferentiated cells in the overlying connective tissue labeled with ³H-thymidine primarily differentiate to chondroblasts (2). Northern blot analysis of the repair tissue from the DBM-treated cranial defects showed that

collagen type I mRNA was present at all times but its expression significantly increased by day 5. Osteocalcin mRNA appeared in small amounts by day 4 and continued to increase over the experimental period. Much lesser quantities of collagen types II and X mRNA appeared only after days 6 and 8, respectively. Analyses of collagen synthesis within the cranial implants by *in vivo* ³H-proline labeling at days 3-7 and cyanogens bromide (CNBr) peptide mapping showed that newly synthesized type I collagen was evident on days 3-7, whereas type II collagen appeared only after 6-7 days (3).

These results demonstrate that DBM directly induces the proliferation and differentiation of mesenchymal stem cells to osteoblasts synthesizing bone matrix when implanted in large (8mm diameter) calvarial defects which do not heal if left untreated. The process is essentially independent of cartilage formation and the sequence of endochondral ossification. In sharp contrast to the repair response induced by the implantation of DBM in cranial defects, implantation of DBM into subcutaneous sites first induced the proliferation and differentiation of the mesenchymal stem cells to cartilage cells and not to osteoblasts. The cartilage cells were subsequently resorbed and replaced by bone and bone marrow (1).

This is the first report that the repair of cranial defects after implantation of DBM or bone morphogenetic proteins (BMPs) occurs initially by the induction of osteoblasts and formation of bone and **not by** the induction of chondroblasts which later undergo the endochondral sequence of ossification as previously reported in the literature (4).

References

1. **Wang J, Glimcher MJ.** Characterization of matrix-induced osteogenesis in rat calvarial bone defects. Part I: Differences in the cellular response to demineralized bone matrix implanted in calvarial defects and in subcutaneous sites. *Calcif Tissue Int* 1999;65:156-165.
2. **Wang J, Glimcher MJ.** Characterization of matrix-induced osteogenesis in rat calvarial bone defects. Part II: Origins of bone forming cells. *Calcif Tissue Int* 1999;65: 486-493.
3. **Wang J, Yang R, Gerstenfeld LC, Glimcher MJ.** Characterization of matrix-induced osteogenesis in rat calvarial bone defects. Part III: Gene and protein expression. *Calcif Tissue Int* 2000;67:314-320.
4. **Damien CJ, Parsons JR, Prewett AB, Rietveld DC, Zimmerman MC.** Investigation of an organic delivery system for demineralized bone matrix in a delayed-healing cranial defect model. *J Biomed Res* 1994;28:553-561.

NOVEL USE OF 3-DIMENSIONAL MAGNETIC RESONANCE IMAGING TECHNIQUE TO STUDY BONE DENSITY

Yaotang Wu, Ph.D.

MAGNETIC RESONANCE IMAGING OF SOLID BONE

One of the most important characteristics of bone tissue and substance is the extent of mineralization, which is quantified as the total volume of the bone material (substance) occupied by the solid Ca-P phase (bone crystals). This critical data will significantly help clinicians evaluate the diagnoses and effectiveness of therapy in patients with heritable and metabolic diseases of the skeleton and with bone defects.

The goal of our bone density project is to develop a chemically selective and three-dimensional Magnetic Resonance Imaging (MRI) technique to measure bone mass and the degree of bone mineralization. More specifically, bone mineral density, defined as $D_p = \text{Weight of Mineral (g)} / \text{Volume of Bone Tissue (cm}^3\text{)}$, can be measured by Solid State ^{31}P MRI. Bone matrix density, defined as $D_H = \text{Weight of Bone Matrix (g)} / \text{Volume of Bone Tissue (cm}^3\text{)}$, can be measured by water and fat suppressed proton projection MRI. The degree of mineralization, $DM = D_p / D_H$ can then be determined from these measurements.

Since the major constituent of bone mineral may be described as a poorly crystalline nonstoichiometric apatite similar, but not identical, to hydroxyapatite, $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$, it is reasonable to assume that quantitative ^{31}P solid state MRI will yield a good representation of the mineral density in bone. In our preliminary studies, we developed a true 3-D solid state ^{31}P MRI technique to measure 3-D volumetric bone density in vitro in a number of different specimens of isolated bones (1).

We have furthered this study to show that ^{31}P solid state MRI is capable of imaging bone in wet whole limbs (with skin, muscle, fascia, subcutaneous tissue, etc.) from animals obtained at an abattoir. Only the mineral components of bone were visible in the ^{31}P image. This study also demonstrated that compact cortical bone and trabecular bone could be independently imaged and measured by solid state MRI (2). In other preliminary experiments, we have succeeded in imaging long bone by ^{31}P MRI in living human subjects (3).

Measurement of 3-D organic matrix density by solid state ^1H MRI is much more difficult than by ^{31}P MRI. In addition to the short T_2 problem common in imaging solid subject, the presence of dominant proton signal from fat and water in bone tissue is a major obstacle to measuring true organic matrix density. Recently we have successfully made considerable progress in accomplishing this measurement.

A preliminary evaluation of quantitative 3-D proton solid state MRI as a means of noninvasively measuring bone matrix (osteoid) density was carried out on bovine bone specimens. In our method, the fluid signal (water and fat) of a trabecular bone specimen is suppressed to yield a solid proton image that represents largely the matrix content. A total image (obtained as in the solid image but without water and fat suppression) gives the total proton content (fluid + solid). The ratio of these two images, after intensity correction against a collagen standard

to compensate for the effects of the suppression sequence and the response to the imaging sequence, yields the mass fraction of matrix. (4)

MAGNETIC RESONANCE SPECTROSCOPY STUDY OF BONE MINERAL

^{31}P solid state nuclear magnetic resonance (NMR) spin-spin relaxation studies were carried out on bovine bone and dental enamel crystals of different ages and the data were compared with those obtained from pure and carbonated hydroxyapatites. By measuring the ^{31}P Hahn spin echo amplitude as a function of echo time, Van Vleck second moments (expansion coefficients describing the homonuclear dipolar line shape) were obtained and analyzed in terms of the number density of phosphorus nuclei. ^{31}P magnetization prepared by a 90° pulse or by proton-phosphorus cross-polarization (CP) yielded different second moments and experienced different degrees of proton spin-spin coupling, suggesting that these two preparation methods sample different regions, possibly the interior and the surface, respectively, of bone mineral crystals. Distinct differences were found between the biological apatites and the synthetic hydroxyapatites and as a function of the age and maturity of the biological apatites. The data provide evidence that a significant fraction of the protonated phosphates (HPO_4^{2-}) are located on the surfaces of the biological crystals, and the concentration of unprotonated phosphates (PO_4^{3-}) within the apatitic lattice is elevated with respect to the surface. The total concentration of the surface HPO_4^{2-} groups is higher in the younger, less mature biological crystals. (5)

IDENTIFICATION OF A CALCIUM-ORGANIC PHOSPHATE COMPLEX AT EARLY STAGES OF MINERALIZATION

Previous ^{31}P cross-polarization and differential cross-polarization magic-angle spinning (CP/MAS and DCP/MAS) solid-state NMR spectroscopy studies of native bone and of the isolated crystals of the calcified matrix synthesized by osteoblasts in cell culture identified and characterized the major PO_4^{3-} and minor HPO_4^{2-} phosphate components of the mineral phase. The isotropic and anisotropic chemical shift parameters of the minor HPO_4^{2-} component in bone mineral and in mineral deposited in osteoblast cell cultures were found to differ significantly from those of brushite, octacalcium phosphate and other synthetic calcium phosphates.

However, because of *in vivo* and *in vitro* evidence that phosphoproteins may play a significant role in the nucleation of the solid mineral phase of calcium phosphate in bone and other vertebrate calcified tissues, the focus of the current solid-state ^{31}P NMR experiments was to detect the possible presence of and characterize the phosphoryl groups of phosphoproteins in bone at the very earliest stages of bone mineralization, as well as the possible presence of calcium-phosphoprotein complexes. The present study demonstrates that by far the major phosphate components identified by solid-state ^{31}P NMR in the very earliest stages of mineralization are protein phosphoryl groups which are not complexed with calcium. However, very small amounts of calcium-complexed protein phosphoryl groups as well as even smaller, trace amounts of apatite crystals

were also present at the earliest phases of mineralization. These data support the hypothesis that phosphoproteins complexed with calcium play a significant role in the initiation of bone calcification. (6)

Previous measurements of the hydroxyl ion content of the calcium phosphate crystals of bone mineral have indicated a substantial depletion or almost complete absence of hydroxyl ions, notwithstanding their presumed status as an integral constituent of the hydroxyapatite lattice. Historically, all analytical methods applied to determine bone crystal hydroxyl content have required chemical pretreatment to eliminate interference from the organic matrix that may have biased the results. This study demonstrates a two dimensional solid state NMR spectroscopy technique which detects the proton spectrum of bone

crystals while suppressing the interfering matrix signals, eliminating the need for specimen pretreatment of any kind other than cryogenic grinding. Results on fresh frozen and ground whole bone of several mammalian species, including rat, bovine and human, demonstrate that the bone crystal hydroxyl ions are readily detectable. A rough estimate yields a hydroxyl ion content of human cortical bone of about 20 percent of the amount expected in stoichiometric hydroxyapatite. This finding holds important implications regarding the biochemical processes underlying normal bone mineral metabolism and metabolic bone diseases such as osteoporosis, as well as the design of bioactive synthetic materials for implanted skeletal prostheses. (7)

References

1. **Wu Y, Ackerman JL, Chesler DA, Li J, Neer RM, Wang J, Glimcher MJ.** Evaluation of bone mineral density using three dimensional solid state phosphorus-31 NMR projection imaging. *Calcif Tissue Int.* 1998;62:512-518.
2. **Wu Y, Chesler DA, Glimcher ML, Garrido L, Wang J, Jiang HJ, Ackerman JL.** Multinuclear solid state three dimensional MRI of bone and synthetic calcium phosphates. *Proc Natl Acad Sci USA.* 1999;96:1574-1578.
3. **Wu Y, Ackerman JL, Chesler DA, Wang JX, Glimcher MJ.** In vivo solid state ³¹P MRI of human tibia in 1.5 T. *Proceedings of the 7th International Society for Magnetic Resonance in Medicine Scientific Meeting.* April, Philadelphia, USA, 1999
4. **Wu Y, Ackerman JL, Chesler DA, Graham L, Wang Y, Glimcher MJ.** Density of organic matrix of native mineralized bone measured by water and fat suppressed proton projection MRI. *Magn Reson Med,* In Press
5. **Wu Y, Ackerman JL, Kim H-M, Rey C, Barroug A, Glimcher ML.** Nuclear magnetic resonance spin-spin relaxation of bone mineral crystals. *J Bone Miner Res.* 2002;17:472-480.
6. **Wu Y, Ackerman JL, Strawich E, Rey C, Kim H-M, Glimcher MJ.** Phosphate ions in bone: Identification of a calcium-organic phosphate complex by 31P solid state NMR spectroscopy at early stages of mineralization. *Calcif Tissue Int.* In Press
7. **Cho G, Wu Y, Ackerman JL.** Detection of hydroxyl ions in bone mineral using solid state NMR spectroscopy. *Science.* In Press