2006 IOF+ANZBMS Combined Meeting

Combined Meeting of the
3rd IOF Asia-Pacific Regional Conference on Osteoporosis *and the*
16th Annual Meeting of the ANZ Bone & Mineral Society
~ 22-26 October 2006, Port Douglas, Australia ~
www.anzbms.org.au/asm/asm2006

SESSION TIME: 1400 - 1530, Wednesday 25 Oct 2006

Oral Abstracts

6B (Basic) - Mechanisms of bone cell function and regulation II

O67

Cascade of gene expression in human osteoclasts indicates early potent induction of chemokine MCP-1 is essential for human osteoclast formation

C.J. Day, S.J.R. Stephens, M.S. Kim, C.L. Magno and N.A Morrison
School of Medical Science, Gold Coast Campus Griffith University, Southport, QLD, Australia

The induction of a range of chemokines has been identified in both human and mouse osteoclastogenesis *in vitro* models. When compared to untreated controls, monocyte chemotactic protein-1 (MCP-1) is highly up-regulated in *in vitro* human osteoclast formation. Within 24 hours exposure to RANKL, MCP-1 mRNA is induced more than 1000 fold in osteoclast precursors. Following induction of MCP-1, calmodulin1 is maximally induced at three days exposure to RANKL. Transcription factors NFAT1 and NFAT2 (thought to be essential for osteoclast formation) reached peak induction six days after MCP-1 induction. MCP-1 receptor CCR2B was 500 fold upregulated by RANKL at 24 hours, providing an autocrine loop.

Inhibition of MCP-1 autocrine loop signalling using both neutralising antibody and dominant negative MCP-1 (7ND) dramatically suppressed osteoclast formation. Neutralising antibody to MCP-1 (4μg/mL) reduced osteoclast formation from 240±16 per cm^2 (control) to 188±17 *($p=0.01$)* while 7ND (50ng/mL) suppressed osteoclast number to 21±1 per cm^2 *($p=4.8x10^{-8}$)*. Not only did 7ND reagent inhibit osteoclast differentiation, it also suppressed mature osteoclast bone resorption activity, reducing dentine resorption from 81±3 to 18±3 pits per dentine slice *($p=0.0002$)*.

Other chemokines and chemokine receptors were also induced in both human models, however to a much lesser extent [1]. These data indicate that MCP-1 is the most potent chemokines involved in human osteoclast formation and mature function. The data are consistent with RANKL inducing an MCP-1 autocrine loop which then sets off a cascade of gene expression, leading to NFAT induction and osteoclast differentiation.

[1] Kim MS, Magno CL, Day CJ, Morrison NA. J Cell Biochem. 2006 Feb 15;97(3):512-8.

O68

Apo2L/TRAIL abrogates the vicious cycle of cancer-induced bone loss by direct effects on cancer cells within the bone microenvironment

L.M. Thai[1], N. Sims[2], A. Labrinidis[1], S. Hay[1], V. Liapis[1], G.J Atkins, D.M. Findlay[1] and A. Evdokiou[1]
[1]Department of Orthopaedics and Trauma, University of Adelaide and Royal Adelaide Hospital, Hanson Institute, Adelaide, South Australia
[2]St Vincent's Institute, Fitzroy, Australia

We have recently shown that recombinant soluble Apo2L/TRAIL prevents breast cancer-induced osteolysis in a mouse model (1). These protective effects on bone are likely to be mediated by the direct apoptotic actions of Apo2L/TRAIL on breast cancer cells themselves within the bone. The purpose of this study was to investigate whether Apo2L/TRAIL abrogates the "vicious cycle" of cancer-induced bone loss by additional effects on osteoclast differentiation and activity. Histomorphometric analysis from Apo2L/TRAIL-treated animals showed no significant changes in any of the bone histomorphometric parameters including trabecular bone volume/total volume, trabecular number, thickness, and separation, osteoclast surface/bone surface or osteoblast surface/bone surface when compared to the untreated animals. Additional *in vitro* experiments we have shown that recombinant Apo2L/TRAIL fails to block osteoclast differentiation and bone resorption in three separate *in-vitro* model systems of osteoclastogenesis. We have shown that Apo2L/TRAIL does not block osteoclastic differentiation and and bone resorption of human peripheral blood mononuclear cells or the murine monocytic cell line, RAW264.7. Both cell models differentiate into bone resorbing osteoclast in the presence of RANKL. Thirdly we have shown that TRAIL has no effect on the bone resorbing activity of mature osteoclasts isolated from human Giant Cell Tumours of bone when plated on dentine slices. Taken together, our results show that the protective effect of Apo2L/TRAIL on breast cancer-induced osteolysis is mainly mediated by the direct actions of Apo2L/TRAIL on cancer cells themselves and not due to any effects on host osteoclast differentiation and/or activity.

References:

1. L M Thai, A Labrinidis, S Hay, V Liapis, S Bouralexis, K Welldon, B J Coventry, DM Findlay and A Evdokiou. Apo2L/TRAIL prevents breast cancer-induced bone destruction in a mouse model. *Cancer Res*. 2006 May 15;66(10):5363-70

O69

An *in vivo* biological response mediated by a rapid-acting, low calcemic analog of 1,25-dihydroxyvitamin D_3

K.M. Dixon[1], S.S. Deo[1], A W Norman[2], I Nemere[3], G.M. Halliday[4], V.E. Reeve[5] and R.S. Mason[1]
[1]Department of Physiology and Bosch Institute, University of Sydney, NSW, 2006, [2]Department of Biochemistry, University of California, Riverside, CA, USA, 92521, [3] Department of Nutrition and Food Sciences, Biotechnology Center, Utah State University, Logan, Utah, USA, [4]Department of Medicine (Dermatology), and [5]Faculty of Veterinary Science, University of Sydney, NSW, 2006.

Vitamin D is produced by exposure of 7-dehydrocholesterol in skin to UV irradiation (UVR). It can be further converted in skin to 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), which has been shown to generate biological responses via 2 pathways – the genomic pathway and a rapid, non-genomic pathway mediated by a putative membrane-associated receptor (1,25D-MARRS[1] and/or a membrane bound form of the nuclear VDR[2]). We investigated the photoprotective effects of $1,25(OH)_2D_3$ in skin, and have clear evidence that these effects also occur via the rapid pathway. In addition to vitamin D production, UVR results in DNA damage producing cyclobutane pyrimidine dimers (CPD), and increased p53 tumour suppressor protein expression. We previously reported that $1,25(OH)_2D_3$ protects human skin cells from UVR-induced apoptosis and decreases CPD. A *cis*-locked, rapid-acting agonist $1,25(OH)_2$lumisterol$_3$ (JN) mimicked the actions of $1,25(OH)_2D_3$ to increase p53 expression, reduce CPD damage and keratinocyte loss after UVR. These effects were abolished by a rapid-acting antagonist but not by a genomic antagonist. Furthermore, the protective effects were abolished by a neutralizing antibody to the 1,25D-MARRS protein. Apoptotic sunburn cells in Skh:hr1 mice exposed to solar-simulated UVR and treated topically with $1,25(OH)_2D_3$ or JN immediately after UVR were reduced 24h later from 2.7 ± 0.4 to 1.0 ± 0.3 ($p<0.01$) and 1.2 ± 0.1 ($p<0.01$) per 1000μm respectively. Topical $1,25(OH)_2D_3$ and JN reduced CPD measured 24h post UVR from $9 \pm 3\%$ to $3 \pm 1\%$ ($p<0.01$) and $4 \pm 2\%$ ($p<0.01$) respectively. Both compounds significantly reduced systemic UVR-induced immunosuppression from $23 \pm 1\%$ to $5 \pm 1\%$ ($p<0.001$) and $-3 \pm 2\%$ ($p<0.001$) respectively. The data support the hypothesis that $1,25(OH)_2D_3$ exerts its photoprotective effects via the rapid response pathway and that the intrinsic photoprotective properties of skin may be enhanced by low calcemic analogs of $1,25(OH)_2D_3$. These results show for the first time an *in vivo* biological response mediated by a rapid-acting analog of vitamin D.

1. Nemere *et al.*, PNAS 101:7392, 2004
2. Mizwicki *et al.*, PNAS 101:12876, 2004

O70

M-CSF potentiates osteoclastic bone resorption via increased activation of ERK, AP-1 and NFκB

J.M. Hodge, M.A. Kirkland and G.C. Nicholson
The University of Melbourne, Department of Clinical and Biomedical Sciences: Barwon Health, Geelong, Victoria 3220, Australia.

The proliferation and differentiation of osteoclast (OC) precursors is dependent on M-CSF. M-CSF also regulates OC survival, motility and cytoplasmic spreading, although reported actions on resorptive function are contradictory. This study aimed to investigate the role of M-CSF on the survival and function of mature human OC.

Human OC were generated by culturing CFU-GM-derived precursor cells with sRANKL (125ng/mL) and hM-CSF (25ng/mL) for 14-21d. To quantify their resorbing activity, multinucleated OC were removed from the plastic substrate using a non-enzymatic dissociation buffer, settled onto dentine slices and cultured for 72h. Activation of NFκB and AP-1 was assessed using transcription factor assays and IκBα and ERK by Western analysis in OC serum-starved for 18h prior to addition of treatments.

Neither M-CSF nor RANKL were required for mature OC survival, although RANKL was required for resorptive function. Co-treatment with M-CSF had a biphasic action promoting maximal resorption (+181%) and actin ring formation (+131%) at 25ng/mL. M-CSF potentiated RANKL-induced activation of AP-1 and NFκB by 147% and 42%, respectively, but did not augment phosphorylation of IκBα. Activation of ERK by RANKL was poor compared to M-CSF alone (-96%) but in combination yielded an increase of 67% following 10m of stimulation.

We have shown that M-CSF augments RANKL-induced resorbing activity of mature human OC. The mechanism of this effect remains to be determined although we propose a convergence of signaling from RANK and fms via ERK upstream of AP-1.

O71

Deletion mutants of RANKL as potential antagonists in osteoclast differentiation, bone resorption and signaling activation

C. Wang, N. Pavlos, M-H. Zheng, J.W-Y. Tan, J.M. Li[1], J. Cornish[1] and J. Xu*
Molecular Orthopaedic Laboratory, School of Surgery and Pathology, University of Western Australia, Nedlands WA 6009, Australia; Department of Medicine[1], University of Auckland, New Zealand

Receptor activator of NF-κB ligand (RANKL) is a key factor necessary for osteoclast differentiation and activation. In this study, we investigated the biological activity of RANKL mutants and their potential inhibitory action on RANKL-induced formation and bone resorption. To this end, a series of truncated regions of the TNF-like core domain of rat RANKL (rRANKL) were cloned into the bacterial expression vectors pGEX. Soluble forms of recombinant RANKL were affinity-purified and their biological activities assessed using RAW 264.7 cell cultures and isolated rat osteoclast cultures. RANKL mutants (aa160-302), (aa160-268), (aa239-318) and (aa246-318) showed inhibitory effects on osteoclast formation and bone resorption. GST-pulldown protein-protein interaction assays revealed that the same RANKL mutants bind differentially to RANK. In addition, RANKL mutants impaired the activation of NF-κB, IκB-α degradation, c-Jun N-terminal kinase and ERK phosphorylation. Interestingly, RANKL mutants (aa160-302), (aa160-268), (aa239-318) and (aa246-318) competitively inhibited wild type RANKL-induced osteoclastogenesis with RANKL mutant (aa246-318) exhibiting the most potent inhibitory action. RANKL mutant (aa246-318) also suppressed RANKL (aa160-318)-induced osteoclastic bone resorption in isolated rat osteoclast cultures. Preliminary labeling studies revealed that upon binding to RANK, RANKL is internalized and that this process is reduced in the presence of either RANKL mutant (aa246-318) or OPG as evidenced by confocal microscopy and flow cytometric analysis. Collectively, our data indicate that RANKL mutants effectively impair osteoclast differentiation and activation; thus offering potential therapeutic value for the treatment of osteolytic disorders.

O72

Transforming growth factor-β inhibits osteoclast formation in bone marrow-derived macrophages

R. Lari [a], P.D. Kitchener[b] and J.A. Hamilton[a]
[a] *Department of Medicine and CRC for Chronic Inflammatory Diseases, University of Melbourne, The Royal Melbourne Hospital, Parkville, Victoria 3050*
[b] *Department of Anatomy and Cell Biology, University of Melbourne, Parkville, Victoria 3010*

General and local osteoporosis are two major pathological symptoms of rheumatoid arthritis (RA) that are the consequence of osteoclast induction. Transforming growth factor-β (TGF-β) is an abundant growth factor in bone that its expression is highly up regulated in RA. The effects of TGF-β on bone are quite complex although usually found to be direct stimulatory for osteoclast differentiation. To gain better understanding of the role of TGF-β in osteoclastogenesis, we have investigated effects of this factor on osteoclast differentiation of M-CSF-derived macrophages and GM-CSF-derived macrophages *in vitro*. Unexpectedly, we observed that TGF-β potently suppressed M-CSF + RANKL-driven osteoclast differentiation from both cell populations. Also TGF-β significantly suppressed the proliferation of osteoclast precursors ($p < 0.01$) and inhibited osteoclastogenesis in a dose dependent manner. Although, TGF-β did not affect expression of the RANK and *c-fms* genes, it suppressed the induction of the *c-fos* gene (an essential transcription factor for osteoclast differentiation) by M-CSF and RANKL. Using cells from the respective gene deficient mice, this inhibition by TGF-β appeared to be independent of endogenous interferon α/β, GM-CSF and IL-10 production. Our data suggests that the influence of TGF-β on osteoclastogenesis depends upon the maturation state of the osteoclast precursor and on the presence or otherwise of other stimuli such as RANKL and it may represent a potent tool for preventing loss of bone mass in chronic inflammatory disorders, especially in RA.

O73

Maintenance of adult stem cell self-renewal with heparan sulfate

T. Helledie[1], C. Dombrowski[1], I.L.H. Hin[1], H.W. Jin[1], V. Nurcombe[1,2] and S.M Cool[1,2]
[1]Institute of Molecular and Cell Biology, Proteos, 61 Biopolis Drive, Singapore 138673. [2]Department of Orthopaedic Surgery, Yong Loo Lin School of Medicine, National University of Singapore, 119074

Therapies that seek to utilize adult human mesenchymal stem cells (hMSCs) are hampered by insufficient numbers of these rare cells. However, the long-term ex vivo expansion of these cells that is necessary to attain therapeutic numbers directly correlates with a loss of multipotentiality due to a change in the microenvironment[1]. Heparan sulfate (HS), a key component of the stem cell microenvironment, is known to protect growth factors from degradation and is necessary for the formation of specific activating receptor complexes [2,3]. Here we show that a specific HS (HS-2), purified for its ability to potentiate the effects of fibroblast growth factor-2 (FGF-2)[4], can, when added, significantly increase the expansion of hMSCs in an uncommitted state. Upon exposure to HS-2, hMSCs are stimulated to enter the cell cycle, resulting in a 8-fold increase in cell number and resultant colony forming units (CFU-Fibroblastic) after three weeks in culture without a loss of multipotentiality. Cell surface marker and gene expression profiling were then used to monitor the effect of HS-2 on long-term cultures of hMSCs, with the resulting stem cell signature showing that HS-2 protects against a temporal loss of stemness. Thus HS offers a novel means for potentiating the self-renewal of stem cells that is independent of exogenous applications of growth and adhesive factors that can otherwise compromise stem cell fate.

1. DiGirolamo, C.M, *et al.* Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br.J. Haematol*. 107:275–281 (1999).
2. Rapraeger, A.C., Krufka, A., Olwin, B.B. Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science* 252(5013):1705-8 (1991).
3. Yayon, A., Klagsbrun, M., Esko, J.D., Leder, P., Ornitz, D.M. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* 64(4):841-8 (1991).
4. Nurcombe, V., Ford, M.D., Wildschut, J.A., Bartlett, P.F. Developmental regulation of neural response to FGF-1 and FGF-2 by heparan sulfate proteoglycan. *Science* 260(5104):103-6 (1993).

O74

Micro-CT bone morphometric evaluation of post-ovariectomy distal femoral bone loss in three mouse strains

P. Salmon[1], G. Kerckhofs[1], L. Oste[2], L. Van Rompaey[2] and G. Dixon[2]
1. Skyscan, Aartselaar, Belgium
2. Galapagos, Mechelen, Belgium

Aims: The rodent ovariectomy (OVX) model is a standard tool in preclinical osteoporosis research. This study aimed to validate the use of micro-CT morphometry to assess the skeletal outcome of OVX in terms of trabecular and cortical morphometry. Three widely used mouse strains were studied, to allow comparison of their utility as osteoporosis models.

Method: Twelve mice each of three strains, BALBc, C3H/HeN and C57BL/6J, were divided into two groups (n=6) for OVX and sham operation, which were carried out at - 6 weeks. The mice were sacrificed five weeks after surgery, and the left femurs were harvested, fixed and stored in ethanol. A 4.5 mm length of the distal femurs was scanned subsequently by micro-CT (Skyscan 1172) at a pixel size of 4.5 microns. A metaphyseal trabecular region and a diaphyseal cortical region were selected for morphometric analysis, both referenced to the growth plate.

Results: In the BALBc and C3H/HeN strains OVX caused a significant expansion of the metaphyseal volume, but not in C57BL/6J. Trabecular percent volume (BV/TV) decreased following OVX by at least half in C3H/HeN and C57BL/6J compared to shams ($p<0.001$) but by a lesser degree in BALBc ($p<0.01$). This loss of trabecular volume was mostly due to a decreased trabecular number – the changes in Tb.N and their statistical significance mirrored those of BV/TV. Trabecular thickness was significantly reduced by OVX only in

C3H/HeN mice ($p<0.0001$). Trabecular pattern factor and Euler connectivity density showed reduced trabecular connectivity in C3H/HeN and C57BL/6J ($p<0.01$-0.001) but no significant change in BALBc. Fractal dimension showed a fall in trabecular surface complexity in C3H/HeN and C57BL/6J ($p<0.05$-0.001) but not in BALBc, due to OVX. Structure model index showed a change to more rod-like trabeculae in C3H/HeN and C57BL/6J ($p<0.01$-0.001) but again not in BALBc. Cortical periosteal diameter expanded following OVX in BALBc and C3H/HeN ($p<0.05$) but not in C57BL/6J. Cortical thickness was reduced significantly by OVX only in the C3H/HeN mice ($p<0.01$). Cortical porosity increased sharply in the BALBc and C3H/HeN in the OVX groups ($p<0.01$-0.001), but was unchanged by OVX in the C57BL/6J mice.

Conclusion: Micro-CT morphometry showed highly significant changes in trabecular and cortical bone to be caused by OVX in mice. There were significant differences in response between the three strains. Overall the strain apparently best reflecting human osteoporotic changes, in both trabecular and cortical bone, was the C3H/HeN mouse. To further validate this mouse OVX model as an alternative for the commonly used rat model, further studies to test efficacy of estradiol and bisphosphonate are ongoing.