INTRODUCTION: Wear particle-induced granulomatous inflammation has been identified as the major cause of osteoclast-mediated peri-implant osteolysis. Osteoclasts, the principal bone-resorbing cell type, are derived from monocyte macrophage lineage cells which are recruited in response to wear debris. Macrophages differentiate into pre-osteoclasts under the influence of the cytokine milieu and RANKL, the primary mediator.

Gene expression and hence phenotype is dramatically influenced by the matrix that a cell adheres to. Analysis of retrieved peri-implant tissues demonstrates a critical role for the bone surface in determining the differential genetic program and phenotypic features of macrophage polykaryons. In peri-implant osteolysis, macrophage on particulate wear debris differentiate to foreign body giant cell polykaryons, while bone-adherent cells differentiate to mature resorptive osteoclast polykaryons [1]. Recently the cluster of bone matrix-adherence dependent osteoclast genes was identified and annexin A8 was described as a marker for this cluster. annexin A8 was shown to be a critical osteoclast gene that is specifically and significantly up-regulated by bone-adherent, resorbing, osteoclasts both in vitro in peri-implant tissues and in vivo [2].

The integrin β3, as the αvβ3 integrin heterodimer, is highly expressed on bone-resorbing osteoclasts and is required for normal osteoclast formation and function [3]. The αvβ3 integrin mediates osteoclast cell adhesion to bone proteins via an RGD (Arg-Gly-Asp) motif. The β3 integrin gene is induced by RANKL through the transcription factor NFATc1 during osteoclast differentiation [4].

In the present studies we asked if expression of the bone matrix-adherence dependent osteoclast genes are down-stream of and, in turn, dependent upon expression of the β3 integrin. Utilizing an in vitro model of osteoclast differentiation with osteoclast precursors from β3 null animals cultured on bone substrates, we have assessed the requirement for signaling via the β3 integrin for expression of the bone matrix-adherence dependent osteoclast gene cluster. In addition, we have identified clusters of genes which require signaling via the β3 integrin for down-regulation in osteoclast differentiation.

METHODS: In vitro culture; mouse bone marrow macrophage (BMM) were isolated as described [4]. Mouse 1° BMM were grown for 5 days with 20 ng/ml RANKL, and 20% MCSF (R&D Systems) plated on discs of mouse calvarial bone or on tissue culture-treated plastic dishes (Costar). RNA was isolated by Trizol extraction using manufacturers instructions (Invitrogen) and reverse transcribed with RETROscript (Ambion). For cells cultured on mouse calvarial bone, 4mm calvarial bone discs were devitalized by several freeze-thaw cycles and treated overnight with collagenase type I at 37°C (Worthington). RNA was prepared from cells on 3-4 discs combined for Trizol extraction for each replicate. In parallel cultures, cells were fixed with 4% paraformaldehyde (PFA) and stained for TRAP and actin ring formation with rhodamine tagged phalloidin (Molecular Probes). Real-time RT-PCR (QPCR) was performed for osteoclast marker genes including tartrate-resistant acid phosphatase (TRAP), cathepsin-k, the calcitonin receptor (CTR), and annexin A8 using the Mx3000P QPCR System (Stratagene) and SybrGreen Master Mix (ABI). Gene expression was normalized to HMBS gene expression using the 2-ΔΔCT method. RNA prepared from these cultures was subjected to oligonucleotide array analysis profiling on Affymetrix Mouse Genome 430 2.0 GeneChips, for analysis of over 39,000 transcripts and variants. Following microarray normalization and gene comparison, hierarchical clustering was performed on the subset of genes showing the greatest amount of variation using the GeneSpring GX program. In parallel cultures, cells were fixed with 4% paraformaldehyde (PFA) and stained for TRAP and actin ring formation with rhodamine tagged phalloidin (Molecular Probes).

RESULTS: As an in vitro model, amenable to bio-molecular analysis, osteoclast precursors cells from wild type and β3 null animals were cultured on tissue culture plastic or on mouse calvarial bone for 5 days with MCSF and RANKL. We find that TRAP positive multinuclear cells form in all cultures under these conditions. As expected [3], osteoclasts in cultures derived from β3 integrin null animals were not spread on either tissue culture plastic surfaces or on mouse calvarial bone discs. Staining with rhodamine-conjugated phalloidin showed strong actin ring formation in wild type osteoclasts cultured on calvarial bone discs while β3 integrin null osteoclasts showed limited actin organization, again as expected [3].

We measured osteoclast marker gene expression by QPCR to validate the RNA samples and to ensure osteoclast differentiation. We find that cathepsin-k and TRAP expression were at similar levels independent of matrix and the β3 integrin and, as previously reported, annexin A8 is induced by culture on bone in the β3 integrin null osteoclasts as well as in wild type [5].

Profiling of β3 null osteoclasts on plastic and on bone versus wildtype osteoclasts revealed several interesting gene clusters. When β3 null osteoclasts on bone were compared with ruling wild type osteoclasts on bone, 173 genes were found to be down-regulated in β3 relative to wild type. Notably, “predicted gene EG633640” was 86 fold down in β3 versus wild type, Uch1 was 53.5 fold down, and an unknown transcribed locus (weakly similar to XP_001751121.1) was 30.8 fold down. The β3 integrin was seen among the down-regulated in β3 null versus wild type cluster, as expected.

Overall, matrix-dependent down-regulation in β3 null osteoclasts was only 73% of that seen in wild type osteoclasts on average (range = 44.2% to 143%). Gene up-regulation in β3 null osteoclasts on bone versus plastic is also 73.5% of that seen in wild type osteoclasts on average (range = 24.8% to 292%). Of the 112 genes which were up-regulated in β3 null on bone versus plastic, 3 are found down-regulated in wild type osteoclasts on bone versus plastic (Prg4, Ibsp, and Hal). Of the 25 down-regulated in β3 null on bone versus plastic, 3 are found to be up-regulated in wild type osteoclasts on bone versus plastic (Krtap5-2, Kbbd11, and Serpine 2).

DISCUSSION: Interaction of the bone surface is critical for gene expression, morphology, and phenotype of a fully functioning osteoclast. Our findings indicate the essential role of β3 integrin-mediated cell-substrate interactions in regulation of the genetic program associated with the terminal osteoclast differentiation and activation. We also identify clusters of osteoclast genes which are specifically up-regulated in osteoclasts on bone and are dependent on β3 integrin expression and clusters which are independent of β3 integrin expression. These findings have important implications for the development of therapeutic strategies that target specific osteoclast genes and signal activation pathways for the prevention of pathologic bone loss.

REFERENCES: