A Single Antibody based ELISA for the N-terminal sequence of BAG-75, a New Biomarker for Bone Formation

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Bone acidic glycoprotein-75 (BAG-75) is a secreted product of osteoblastic cells localized predominantly to areas of new bone formation. We have identified the N-terminal sequence of BAG-75 as LPVARYQNTEEEE and shown that anti-peptide antibodies against residues #3-13 only recognize the 75 kDa precursor and apparent 50 kDa fragment in serum and in osteoblastic cultures. Formation of the 50 kDa fragment is blocked by AEBSF, a serine protease inhibitor which we also showed blocks mineralization in osteoblastic cultures. Measurement of BAG-75 and its fragment concentration in serum represents a new method to estimate the rate of new bone formation in vivo. Our purpose was to establish an anti-VARYQNTEEEE peptide antibody based ELISA test to measure cross-reactive proteins released from bone into blood. Western blotting was performed using young rat serum from different ages, rats subjected to ovariectomy (OVX) or sham surgery, and normal human serum. Immunoreactive 50 kDa fragment peaked at 18 days after birth which parallels bone formation. Ovariectomized rats displayed a peak of 50 kDa immunoreactivty at 21 days after surgery which corresponds to a spike in bone formation in this model (~2.5-fold above controls). Comparable assays for osteocalcin showed only a 39% increase. Also, human serum contains a 50 kDa protein which cross-reacts with anti-VARYQNTEEEE antibodies. We then established a competitive 96-well ELISA using anti-peptide antibody and new sera at 21 days from ovariectomized or sham rats, a model for stimulated bone formation. VARYQNTEEEE peptide conjugated to keyhole limpet hemocyanin (KLH) was used as the bound antigen. KLH-peptide amount, primary antibody concentration, secondary antibody concentration, and blocking

agent were optimized in a series of experiments. Optimal conditions were determined to be 2 µg input KLH-peptide per well, 1/5,000 dilution of primary anti-VARYQNTEEEE antibody, 1/10,000 dilution of secondary antibody, and gelatin as a blocking agent. Sera from OVX rats and sham-operated controls were compared to the standard curve (r = 0.9923) created with free KLH-peptide as competitor to determine the equivalent amount of KLH-peptide present. OVX sera (n=3) contained an average 2.6×10^{-4} (+/- 1.4×10^{-4}) µg peptide equivalent versus 1.05×10^{-4} (+/- 0.68×10^{-4}) µg for sham sera (n=3). The difference was not significant (t-test, p=0.157), however, doubling the sample size is predicted to yield significance. **Conclusions**: A. Cross-reactive 75 kDa and 50 kDa proteins are present in human and rat serum and increase in concentration when bone formation is stimulated. B. A new, single antibody based ELISA assay was established to quantitate antigen released from bone into blood. C. In contrast to other commercial bone formation assays (collagen peptides and osteocalcin), the size of cross-reactive protein (>50 kDa) should preclude kidney filtration and facilitate measurement. D. This serum biomarker undergoes a 2-3 fold average increase within 3 weeks after simulation of bone. This test may be useful to monitor the early response to stimulatory therapy in osteoporosis patients or to repressive glucocorticoid therapy in sarcoidosis patients. Currently, a 1% change in bone mineral density requires 12-18 months to detect by x-ray methods.