RADIATION & CHEMOTHERAPY REDUCE OSTEOCLASTIC RESORPTION IN VITRO

+*Margulies, BS; *Schoonmaker, JE; *Strauss, JA;*Damron, TA;*Allen, MJ
*Upstate Medical University, SUNY, Syracuse, NY

Introduction: The treatment of childhood malignancies may result in an inability to obtain peak bone mass during development that in turn may lead to osteoporosis; one of several late complications observed in pediatric patients following irradiation and chemotherapy. We have developed two in vivo Sprague-Dawley rat models to separately ascertain the effects of bone cancer therapies.1,2 In the first, the chemoradioprotectant amifostine was employed to reduce the negative radiation therapy effects on growth. In the second, high dose methotrexate therapy induced osteopenia was corrected with the anti-osteoporosis drug alendronate. In both models, bone density increases were observed that likely reflect treatment effects on osteoclasts, the cells responsible for bone resorption. To determine treatment dependent differences in osteoclast activity we developed a quantitative method called the clast index (CI) that relies in part on the measurement of the enzyme tartrate resistant acid phosphatase (TRAP). The CI has allowed for the quantification of individual osteoclast bone resorptive capacity in un-decalcified sections of rat bone, for which micro-regional differences in osteoclast number and morphology were successfully demonstrated. To validate our previous work we employed an established in vitro model and a panel of standard resorption assays in combination with the assay for the 5b isofrom of TRAP, a product of osteoclast activity related to bone resorption.3 We hypothesize that treatment with radiation and chemotherapy will lead to decreased TRAP expression; reflecting decreased osteoclast resorptive capacity corroborated in the other bone resorption assays employed in this study and previous in vivo work.1,2

Methods: Using an IRB approved protocol; human bone marrow was collected from adult patients undergoing elective total hip arthroplasty. Monocytes were separated from whole bone marrow aspirates and their numbers were enriched through the addition of 25 ng/ml of macrophage colony stimulation factor (M-CSF). Osteoclasts were derived from 1x10⁶ enriched monocytes supplemented with 25 ng/ml of M-CSF and 30 ng/ml of the ligand for the receptor activator for nuclear factor kappa-beta (RANK-ligand). Two experiments were performed to validate the CI to other known markers of osteoclast resorption: In the first, the temporal profile of TRAP 5b expression during osteoclast development was determined in cultures supplemented with the following factors that promote osteoclast formation: 25 ng/ml M-CSF, 25 ng/ml M-CSF plus 30 ng/ml RANK-ligand, 1 µM 1, 25 dihydroxyvitamin D, 10 nM dexamethasone, or 1 µM of vitamin D plus 10 nM of dexamethasone. In the second group of experiments TRAP 5b expression was compared to other known resorption assays following treatment: 0 and 0.5 mM amifostin was administered 20 minutes prior to serial doses of 0, 2 and 5 Gy x-irradiation therapy using a Phillips-MGC-30 radiotherapy unit operated at 300 kV and 10 mA (dose rate=2.09 Gy/min, 15 cm source-object distance). In addition, the bisphosphonate alendronate was administered to cells at 0, 0.1, 1, 10, 100 and 1000 µM concentrations.1

At 21 days following treatment, staining for the osteoclast specific markers vitronectin receptor and TRAP were used to confirm the osteoclast phenotype for counting. Resorption was measured directly by sampling culture media from osteoclasts grown on bone slices using the TRAP 5b assay (pH 6.1), and the cross-linked N-terminal telopeptide of type I collagen (NTX) competitive ELISA kit. Bone slices were then processed for the pit-formation assay and TRAP histochemistry. Computer-based image analysis was used to measure the total area of the resorption pits. Separate bone slices were fixed for TRAP staining, embedded in PMMA and serially sectioned at 5 µm. The CI was determined from the cells on bone slices using the following: the area of the cell in section (A), the length of the cell resorptive interface with the bone slice (R), and the area within the cell occupied by TRAP stained granules (T), such that CI=(R*T)/A. Statistical analysis was performed using ANOVA and Fisher's PLSD post hoc test (p<0.05).

Results: The temporal expression of TRAP 5b during osteoclast development at 18 days following the addition RANK-ligand produced a statistically significant increase of 55% over M-CSF controls (p<0.01); with RANK-ligand producing the greatest increase in TRAP 5b expression relative to all other factors tested. Irradiation therapy resulted in statistically significant decreases of 53 and 78% in TRAP 5b expression (Δ, p<0.001) and decreases of 75 and 88% in pit formation (Δ, p<0.001) for 2 and 5 Gy irradiation relative to non irradiated controls. (Figure 1 & 2) Further statistically significant decreases of 88, 96 and 97% in TRAP 5b expression for the 0, 2 and 5 Gy amifostine treated cultures were observed relative to the non irradiated controls (*, p<0.001). (Figure 1) Corresponding statistically significant decreases of 58, 80 and 90% in pit formation were observed for the 0, 2 and 5 Gy amifostine treated cultures were observed relative to the non irradiated controls (*, p<0.001). (Figure 2) In cultures that were irradiated, osteoclasts (arrows) had smaller resorption interfaces than osteoclasts in non irradiated cultures (arrow heads in 0 Gy). (Figure 3) Following amifostine plus irradiation therapy, both the resorption interface and the intensity of the TRAP staining decreased. (Figure 4) NTX values possessed similar trends to the TRAP 5b expression and pit formation values. Treatment with alendronate resulted in statistically significant reductions of 14, 22, 83 and 93% in TRAP 5b expression for the 1, 10, 100 and 1000 µM doses (p<0.01). Regression analysis was performed for TRAP 5b relative to NTX (r²=0.81, p<0.02) and TRAP 5b relative to pit formation (r²=0.75, p<0.025).

Discussion: The TRAP 5b media assay correlated very well with the NTX assay and the pit formation assay. Qualitatively, the decreased resorption interface observed following irradiation and the decrease in both resorption interface and TRAP expression following amifostine and irradiation therapy suggest that the CI would follow a similar trend quantitatively. Further, we observed in vitro that irradiation, irradiation plus amifostine and alendronate treatment correlate very well with our in vivo Sprague-Dawley animal model data.1,2 Further work is needed to ascertain the 3-dimensional morphological characteristics of osteoclasts and the corresponding CI.


Acknowledgements: Nation Osteoporosis Foundation