Thermally Induced Osteocyte Apoptosis Initiates Osteoclastic and Osteoblastic Responses

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Introduction: During orthopedic procedures, bone and the surrounding soft tissue can be exposed to elevated temperatures arising from surgical cutting and exothermal reactions from cement and biomaterials. Our recent study has shown that temperatures exceeding 47°C can trigger in vitro cellular responses, such as osteocyte apoptosis [1]. Micro-damage induced osteocyte apoptosis in vivo can trigger pro-osteoclastogenic signals that initiate osteoclastic bone resorption [2]. However, whether thermally induced osteocyte apoptosis can initiate expression of osteoclastogenesis remains to be determined. Furthermore, we have also shown that markers related to osteogenesis (alkaline phosphatase activity and calcium deposition) were up-regulated in Mesenchymal Stem Cells (MSCs) when directly exposed to such temperatures [1]. However, it is not yet known whether heat-induced osteocyte apoptosis can elicit osteoblastic differentiation of neighboring MSCs. The aim of this study is to examine the influence of secreted factors from heat-treated osteocyte-like MLO-Y4s on the activation of preosteoclast-like Raw 264.7 cells and Balb/c MSCs. This is investigated using a co-culture system, which exposes the Raw 264.7 cells and MSCs cultured on permeable well inserts to biochemical molecules produced by the heat-treated MLO-Y4’s, whereby the Raw 264.7 cells and MSCs do not undergo heat-treatment themselves.

Methods: Heat-treatment: MLO-Y4 cells were cultured on collagen coated slides. Balb/c MSC’s (ccMSC) and Raw 264.7 (ccRAW) cells were cultured on permeable inserts (PET 1 μm pores; Millipore, Cork, Ireland). All cells were seeded at a density of 5 x10^4 cells/cm2 and cultured for 24 hours. MLO-Y4’s cells were exposed to preheated media at either 37°C (control), 47°C or 60°C, and maintained on a hot plate at these temperatures for 1 minute. Heat-treated MLO-Y4’s were returned to the incubator for 30 minutes to allow the cells to reach to the normal cell culture temperature of 37°C. Untreated Balb/c MSC’s or Raw 264.7 cells on inserts were then added to the wells of the heat-treated MLO-Y4s and co-cultured for up to 21 days. Osteoclastic Differentiation Markers: Receptor activator of nuclear factor kappa-B ligand (RANKL) activity of the MLO-Y4s was determined by ELISA (Mouse RANKL ELISA, R and D Systems). Pre-osteoclast maturation was also quantified in terms of intracellular Tartrate resistant acid phosphatase (TRAP) activity of ccRAW cells using cell lysate measured colorimetrically with p-nitrophenyl phosphate as a substrate (Abcam, UK). Osteoblastic Differentiation Markers: Intracellular alkaline phosphatase (ALP) activity was measured using cell lysate measured colorimetrically with p-nitrophenyl phosphate as a substrate (Sigma) for ccMSCs. Mineralization of ccMSCs was also quantified by alizarin red staining, whereby the stain was extracted using cethylpyridium chloride and the solution measured at 550nm using a spectrophotometer. Cell Proliferation: Cell lysate was measured for DNA content for all cell types using the fluorescent dye Hoechst 33258 (Sigma). Statistical Analysis: Statistical differences between groups were determined using an ANOVA-crosed factor model, Tukey’s test method for comparison between treatments at each time point was used to determine statistical significance, p≤0.05 (MINITAB v.16).

Results: Osteoclastic Differentiation: Direct exposure of MLO-Y4 cells to 47°C causes a significant increase in RANKL production per picogram of DNA at day 4 (p=0.0002), compared with the control, see Figure A. A significant initial increase in TRAP activity per picogram DNA is seen at day 4 in ccRAW cells cultured with MLO-Y4s heat-treated to 60°C (p≤0.0001) compared to the control, see Figure B. Osteoblastic Differentiation: Intracellular ALP expression and calcium deposition by ccMSCs shows a progressively increasing trend for MSC cells cultured with heat-treated MLOY4s. ALP expression per picogram DNA is significantly higher in ccMSCs cultured with MLOY4s heat-treated to 47°C at day 7 (p≤0.0001) compared with control, see Figure C. Similarly ALP expression is higher in ccMSCs co-cultured with heated treated MLOY4s (47°C,60°C) by day 14 (p≤0.0001) compared with control. Calcium deposition is significantly higher in ccMSCs exposed to the biochemical factors produced by MLOY4s heat-treated to 47°C at 21 days compared with the control, see Figure C.

Discussion: This study establishes the link between osteocyte thermal damage and the bone remodeling cascade. Our results indicate that heat-treatment of osteocytes in vitro initiates an early response (4 days after heat treatment) involving the production of pro-osteoclastogenic signaling molecules (RANKL). Additionally, TRAP activity is subsequently increased in ccRAW cells at day 4 when co-cultured with heat-treated MLOY4s. Moreover, the pro-osteoblastic signaling molecule, alkaline phosphatase, and calcium deposition were not expressed until day 14, indicating a longer-term regenerative response following heat treatment of osteocyte cells above. The sequence of events observed in this study; involving osteoclast activity followed by osteoblastic differentiation, is similar to that occurring during bone remodelling in vivo. Therefore, we propose that heat exposure to osteocytes triggers the bone remodeling cascade. Osteocytes are widely accepted as sensory cells that control and regulate bone remodelling in response to
mechanical stimuli and micro-damage [3]. Previous studies have suggested that micro-damage is detected by neighbouring osteocytes that have the capacity to produce essential cytokine signals (RANKL) to stimulate osteoclast differentiation [2, 4]. Furthermore, it has been reported that soluble factors secreted by osteocytes in response to mechanical stimulation in vitro can enhance osteogenesis by MSCs [5]. In a previous study, we determined that osteocytes immediately experience thermal elevations of the surrounding matrix at their mechanosensitive cell processes, demonstrating the vital role of osteocytes in detecting thermal damage [6]. We have also observed a significant apoptotic cell population when osteocytes were exposed to thermal elevations (≥47°C) [1], outlining their role in signalling thermal damage. The results of the current study suggest that heat-induced osteocyte apoptosis can elicit an initial pro-osteoclastic and later stage pro-osteoblastic response, in a similar fashion to micro-damage induced remodelling.

**Significance:** We elucidate the time-line of initial cell thermal damage to the resorption and regeneration responses in vitro and also confirm the vital role of the osteocytes in detecting and responding to thermal damage. These studies provide a novel insight into the mechanisms by which bone cells respond to thermal elevations associated with orthopedic procedures, initiating the healing process.

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