The Regulatory Roles of Inorganic Phosphate in Growth Plate Chondrocyte Apoptosis

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Introduction: Growth plate chondrocytes undergo a series of differentiation steps with programmed cell death (apoptosis) being the final fate of growth plate chondrocytes. Impaired apoptosis of growth plate chondrocytes results in impaired endochondral ossification. Recent studies suggested that inorganic phosphate (P_i) regulates apoptotic events in growth plate chondrocytes (1,2). Hypophosphatemic mouse models have elongated hypertrophic zones and decreased apoptotic rates in the growth plate resulting in the formation of rickets (2). In addition, in vitro studies have shown that growth plate chondrocytes exposed to increasing amounts of P_i undergo apoptosis (1,2). Apoptosis may also play an important role during pathology of cartilage. For example, previous studies have shown that articular chondrocytes in osteoarthritic cartilage undergo apoptosis (3,4). However, little is known about the mechanisms regulating apoptotic events in chondrocytes and the exact role of P_i in the regulation of these events. The purpose of this study was to determine the mechanism of how phosphate regulates apoptosis of growth plate chondrocytes.

Materials and Methods: Primary growth plate chondrocytes were isolated from d19 embryonic chick tibia growth plate cartilage. Cells were cultured for 1 week in DMEM supplemented with 5% fetal calf serum (FCS). After one week the cells were washed with phosphate buffered saline and cultured in DMEM containing 0% or 5% FCS in the presence of increasing amounts of P_i (1mM, 2.5mM, 4mM, 8mM) and in the absence or presence of 35nM retinoic acid (RA) and phosphoformic acid (PFA; 1mM). After various time points the cultures were analyzed for caspase-3 activity using the ApoAlert Caspase-3 Fluorescent Assay kit (Clontech), TUNEL labeling using the ApoAlert TUNEL Assay kit (Clontech) and flow cytometry, and bcl-2 (anti-apoptotic) and bax (pro-apoptotic) expression using real time PCR analysis.

Results: When growth plate chondrocytes were cultured under serum-free conditions increasing amounts of P_i were protective against apoptosis in the presence of RA. Highest caspase-3 activity was detected in cultures treated for 24h in serum-free medium in the presence of RA and 1mM P_i. Caspase-3 activity decreased with increasing amounts of P_i in a dose-dependent manner. In the absence of RA caspase-3 activity was low under serum-free conditions independent of the P_i concentration. TUNEL labeling and flow cytometric analysis revealed similar results. In the absence of RA ~ 15% of the cells were TUNEL positive independent of the P_i concentration. In the presence of RA the highest percentage of TUNEL positive cells (~50%) was detected in the presence of 1mM P_i. The percentage of TUNEL positive cells declined in the presence of higher concentration of P_i in a dose dependent manner. In addition, the bax/bcl-2 expression ratio was the highest in the presence of RA and 1mM P_i and decreased in the presence of higher amounts of P_i in a dose dependent manner. In the presence of PFA, an inhibitor of phosphate transport proteins, caspase-3 activity increased in cultures treated with RA and 8mM P_i for 24h compared to caspase-3 activity of cultures treated with 8mM P_i and RA. When growth plate chondrocytes were maintained in medium containing 5% FCS and treated for 6 days with RA and various concentrations of P_i caspase-3 activity and percentage of TUNEL positive cells increased with increasing concentrations of P_i.

Discussion: Growth plate chondrocytes undergo a series of differentiation steps before they are replaced by bone. As shown by us and others the ultimate fate of growth plate chondrocytes is apoptosis. Apoptosis in the growth plate is restricted to terminal differentiated growth plate chondrocytes close to the chondro-osseous junction (2,5). Therefore, apoptosis of growth plate chondrocytes has to be tightly regulated. Our findings demonstrate that P_i prevents apoptosis in hypertrophic growth plate chondrocytes, whereas it stimulates apoptosis in terminally differentiated growth plate chondrocytes. Under serum-free conditions RA stimulated apoptotic events of hypertrophic growth plate chondrocytes in the presence of 1mM P_i, whereas higher concentrations of P_i prevented RA-induced apoptosis. Contrary, when growth plate chondrocytes were cultured in the presence of FCS, RA stimulated terminal differentiation events (6). Increasing concentrations of P_i then stimulated apoptosis of these terminally differentiated growth plate chondrocytes. These findings suggest that P_i is protective against apoptosis in less mature chondrocytes (hypertrophic) but stimulates apoptosis in more mature cells (terminally differentiated). In addition, our findings reveal that blocking phosphate transport proteins in hypertrophic growth plate chondrocytes prevented the protective effect of P_i on apoptosis of hypertrophic growth plate chondrocytes, suggesting that P_i is being transported into the cell by phosphate transport membrane proteins and then act as a signaling molecule. Future studies have to determine which signaling pathways P_i activates in hypertrophic and terminally differentiated growth plate chondrocytes leading to protection against apoptosis in hypertrophic growth plate chondrocytes and stimulation of apoptosis in terminally differentiated growth plate chondrocytes.

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