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Apoptosis of Growth Plate Chondrocytes Occurs through a Mitochondrial Pathway

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ABSTRACT

Objective: To determine the role of mitochondria in chondrocyte apoptosis induced by inorganic phosphate (Pi).

Materials and Methods: Chondrocytes isolated from the growth plates of chick embryo tibia were treated with Pi in serum-free media; chondrocyte viability, mitochondrial membrane potential, cytochrome c release from mitochondria, caspase 3 activity, endonuclease activity, and DNA fragmentation were investigated.

Results: Exposure to Pi for 24 hours induced apoptosis in growth plate chondrocytes through a pathway that involved loss of mitochondrial function, release of cytochrome c into the cytoplasm, increases in caspase 3 and endonuclease activities, and fragmentation of DNA.

Conclusions: This study suggests that mitochondria are important players in Pi-induced apoptosis.

KEY WORDS: Growth plate, Chondrocytes, Inorganic phosphate, Apoptosis, Caspases, Endonucleases.

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INTRODUCTION Return to TOC

Cartilage is an avascular tissue characterized by an abundant extracellular matrix rich in type II collagen and high molecular weight proteoglycan. There are two types of cartilage, permanent and transient cartilages, that while having the same embryonic origin follow distinct differentiation pathways, fulfill different functions, and have different fates. Articular, tracheal, and other cartilage structures are classified as permanent cartilage. The cells in these structures maintain a stable phenotype and persist throughout life, except under pathological conditions such as osteoarthritis. In contrast, most of the embryonic cartilaginous skeleton, the epiphyseal growth plates of long bones, the cartilaginous callus formed at fracture sites, and the tissue created during distraction osteogenesis consist of transient cartilage.[1,2] Through a series of maturational changes, this cartilage is gradually replaced by bone without disrupting the integrity of the skeleton.

This process, endochondral bone formation, has been the focus of our studies during the past decade, using the growth plate as a model. In these discs of cartilage, responsible for bone growth and located at the epiphysis of long bones, chondrocytes are present in well-defined morphological zones. From the resting region to the calcified cartilage region, chondrocytes undergo a series of maturational changes; they become hypertrophic and calcify the extracellular matrix. At the cartilage-bone interface, the calcified matrix is invaded by vasculature from the bone marrow; and as a result of the activity of different cellular elements (chondrocytes, chondroclasts, osteoblasts, and vascular endothelial cells) converging into this region, the cartilage is resorbed and new bone is deposited. The fate of chondrocytes in this region of the growth plate has been the subject of extensive research. One possible fate is survival in the bone, possibly as an osteoblast or a mesenchymal cell.[3,4]

Our previous work,[5] and that of other laboratories,[6,7] supports the notion that chondrocytes in the growth plate undergo apoptosis. Apoptosis was first described by Kerr et al[8] as a physiologic form of death. The dying cells are characterized by shrinkage of their cytoplasm, condensation of chromatin, blebbing, and formation of vesicles containing the remnants of the cell (apoptotic bodies) that are engulfed by macrophages. During this highly regulated process, there is activation of proteases that lead to cleavage of enzymes and structural proteins and, ultimately, to DNA fragmentation, the hallmark of apoptosis (Figure 1 ).

Little is known about the regulation of apoptosis in growth plate cartilage. We have found that chondrocytes are sensitive to inorganic phosphate (Pi)[9] and that a rise in this anion's concentration induces apoptosis by stimulating nitric oxide generation and other important downstream events, such as the loss of mitochondrial membrane potential ($\Delta\Psi m$).[10] Interestingly, the levels of Pi used in these studies are similar to the levels of this anion reported in the calcified region of the growth plate.[11,12]

It has been found that phosphate can induce a sudden increase in the permeability of mitochondria, an event referred to as the permeability transition.[13] The permeability transition causes the loss of $\Delta\Psi m$ that leads to swelling of the mitochondrial matrix, rupture of the outer membrane, and release of cytochrome c and other mitochondrial proteins into the cytoplasm, causing activation of caspases and committing cells to death.[14] Although our previous studies revealed that Pi-induced chondrocyte death is associated with the loss of mitochondrial function and the activation of caspases,[10] we did not assess whether this process involved cytochrome c release and endonuclease activation.

Although the regulation of the death process in growth plate cartilage is not well understood, alterations in this carefully controlled mechanism can have catastrophic physiologic and quality of life results, as exemplified by severe chondrodystrophy and dwarfism. Indeed, data suggest that low Pi is the cause of delayed apoptosis (rachitic growth plates) in rickets.[15] Therefore, understanding the regulatory mechanism of chondrocyte apoptosis has important potential in interventional therapies for growth anomalies.

In this study, we further explored the chondrocyte apoptotic pathway activated with Pi treatment by investigating the loss of mitochondrial function, release of cytochrome c into the cytoplasm, activities of caspases and endonucleases, and fragmentation of DNA during the first 24 hours of Pi-induced chondrocyte death.

MATERIALS AND METHODS Return to TOC

Cell Culture

Growth plate chondrocytes from 18-day-old chick embryo tibia were isolated as previously reported[16] and plated for 1 week. During this period, the chondrocytes isolated from the whole growth plate proliferate in suspension while fibroblasts and osteoblasts (if present) attach to the culture dishes. Suspended chondrocytes were subcultured and then grown in Dulbecco's modified high-glucose Eagle's medium (DMEM) containing 10% serum (NUSERUM IV, Becton Dickinson, Bedford, Mass), 2 mM L-glutamine, and 50 U/mL each of penicillin and streptomycin. To facilitate cell attachment, cultures were treated with testicular hyaluronidase (4 U/mL). After 3 days, the cells were exposed to Pi in serum-free medium. The actual concentration of Pi in the serum-free medium was 1 mM, and this value was set as the control. A Pi concentration of 5 mM was used in our experiments by supplementing the serum-free medium with sodium dihydrogen phosphate (NaH_2PO_4). To block the transport of Pi, we used phosphonoformic acid (PFA); and to inhibit endonuclease activity, chondrocytes were treated with aurintricarboxilic acid (ATA). All chemicals unless otherwise stated were obtained from Sigma Chemical, St Louis, Mo.

Assessment of Mitochondrial Function

Chondrocytes were plated on glass coverslips and then exposed to Pi for different time periods. Mitochondrial membrane potential was assessed with the voltage-sensitive dye TMRE (Molecular Probes, Eugene, Ore). This probe accumulates only in functioning mitochondria. The cells were incubated with 50 nM of TMRE in phenol-free DMEM for 15 minutes. The cells were washed and then incubated with Hoechst dye 33342 for 2 minutes to visualize their DNA. The chondrocytes were washed with phenol-free DMEM and then evaluated by differential interference contrast and fluorescent microscopy.

Cell Fractionation and Western Blot Analysis

Chondrocytes were harvested with media and then centrifuged at 400 × *g* for 5 minutes. A cytosolic fraction and a mitochondria-enriched fraction were prepared from this cell pellet as described by Dejean et al.[17] Western blot analysis was performed on approximately 20 µg of protein, using antiserum against cytochrome c (Santa Cruz Biotech, Santa Cruz, Cal). To ensure that equal amounts of protein were loaded onto each lane, the membranes were stained with Ponceau S. The signal was amplified by chemiluminescence (ECL kit, Amersham, Boston, Mass), and the membranes were photographed.

Caspase 3 Activity Analysis

To measure caspase 3 activity, cells were incubated with the specific fluorogenic peptide CPP-2/apopain substrate, Z-DEVD-AFC.[10] Caspase activity was monitored by following the blue to green shift in fluorescence upon release of the AFC fluorophore. The excitation and emission maxima for AFC are 400 nm and 505 nm, respectively.

Endonuclease Activity Analysis

Nuclear protein extracts were prepared and then analyzed for endonuclease activity by a zymographic method described by Boone at al[18] that uses a 12% polycrylamide gel containing 250 µg of calf thymus DNA per milliliter. The gel was silver stained according to the Silver Stain kit protocol (Bio-Rad, Hercules, Cal). The silver stains the DNA incorporated into the gel, and endonuclease activity is identified as a clear spot on a dark background.

Viability Assay

Chondrocyte viability was determined by the MTT assay. The optical density was read at 595 nm with a spectrophotometer plate reader, and the results were normalized to values generated by control cells. This assay is widely used to measure cell death, and in a previous report, we showed that we could monitor chondrocyte apoptosis with this procedure.[10]

DNA Fragmentation

Quantification of chondrocytes undergoing DNA fragmentation was performed with the Cell Death Detection ELISA^{PLUS} kit (Roche Molecular Biochemicals, Indianapolis, Ind). This kit allows immunochemical determination of histone-complexed DNA fragments.

RESULTS Return to TOC

Cells undergoing apoptosis present characteristic functional and morphological changes. Figure 2 shows these alterations in chondrocytes exposed to 5 mM Pi. When the cells were labeled with TMRE, their mitochondria stained as long bright rods that accumulated in the perinuclear region of the healthy cell. Chondrocytes maintained this appearance for the first 4 hours of Pi treatment (Figure 2D). Approximately 8 hours after the initiation of treatment, chondrocytes presented signs of stress and began to loose attachment to the glass coverslips; numerous vesicles could be observed in their cytoplasm (arrowhead in Figure 2B). TMRE staining showed that, while remaining functional, the mitochondria presented a different cellular distribution (red speckles and small rods in Figure 2E). After 24 hours of Pi exposure, chondrocytes had shrunk into smaller cellular masses without loosing their membrane integrity (Figure 2C). Note the diffuse and decreased TMRE staining at this stage, suggesting that mitochondria have lost their membrane potential (Figure 2F). Some of the vesicles in the cytoplasm of these cells contain DNA, the result of endonuclease activity (arrowhead in Figure 2F).

Because the loss of mitochondrial membrane potential can lead to the release of cytochrome c and other mitochondrial proteins from these organelles, we investigated the presence of cytochrome c in the cytoplasm of chondrocytes 24 hours after Pi exposure. Chondrocytes were fractionated, and equal amounts of protein from the cytoplasmic and mitochondrial extracts were submitted to Western blot analysis. Figure 3 shows that removing the serum from the culture medium caused a slight increase in the cytoplasmic levels of cytochrome c. When serum-free media was supplemented with 5 mM Pi, we observed a larger increase in cytochrome c in the cytoplasm fraction. As expected, cytochrome c content was greater in the cell fraction containing mitochondria.

Release of cytochrome c into the cytosol results in caspase 3 activation and starts a cascade of protease activities (caspases and endonucleases) that ultimately leads to DNA degradation and death. We quantified the activity of these proteases in apoptotic chondrocytes. As shown in Figure 4A, supplementing serum-free media with 5 mM Pi for 24 hours caused a significant increase in caspase 3 activity in chondrocytes. When the cells were treated with PFA (an inhibitor of Pi cellular transport) before exposure to Pi, caspase 3 activity remained low. We studied endonuclease activity by zymography and found a similar pattern of changes (Figure 4B).

We next examined the effect of the endonuclease inhibitor ATA on Pi-induced chondrocyte apoptosis. MTT and light microscopy analysis revealed that ATA protected chondrocytes from death in a dose-dependent manner (Figure 5). When 5 mM Pi was present in the culture, we observed numerous apoptotic debris and shrunken chondrocytes (Figure 5B). Treatment with 100 µM of ATA conferred total protection to the chondrocytes against Pi (Figure 5D,E), even though the chondrocytes seemed to have lost attachment to the culture dish (compare Figure 5A with 5D).

The hallmark of apoptosis is the presence of DNA fragmentation without the loss of cell membrane integrity. To identify these fragments, the chondrocytes were lysed, and then the extracts were submitted to a colorimetric assay that detects histone-complexed DNA fragments. As shown in Figure 6, serum removal did not induce significant DNA fragmentation during the first 24 hours in culture. However, supplementing the serum-free media with 5 mM Pi caused a drastic increase in DNA fragmentation, confirming endonuclease activity.

DISCUSSION Return to TOC

Our previous studies have shown that treatment of chondrocytes with 5 mM Pi causes significant death levels within 48 hours, accompanied by the loss of mitochondrial function. Here, we focused on the mitochondrial apoptotic pathway and investigated events occurring in the first 24 hours. We found that the loss of mitochondrial membrane potential occurs between 8 hours and 24 hours of exposure to Pi. At the end of the first day of Pi exposure, we observed the presence of cytochrome c in the cytoplasm, high levels of caspase 3 and endonuclease activities in chondrocytes, and resultant DNA fragmentation. This information adds to our laboratory's current effort to increase the temporal resolution of the cascade described in Figure 1 and create a time line of apoptotic events during Pi-induced chondrocyte death.

Recently, it was revealed that under some circumstances the release of cytochrome c from mitochondria precedes the loss of $\Delta\Psi m$, leading researchers to hypothesize the existence of two mechanisms for the discharge of mitochondrial proteins into the cytosol. The first mechanism, described earlier, involves a permeability transition, loss of $\Delta\Psi m$, and swelling and rupture of the mitochondrial outer membrane.[19,20] In the alternative mechanism, the inner membrane potential is maintained, outer membrane remains intact, and cytochrome c is released through the formation of a mitochondrial apoptotic-induced channel, MAC[17–21], with the loss of $\Delta\Psi m$ occurring later in the process.

By creating a time line of events during Pi-induced chondrocyte apoptosis, we will learn whether the discharge of cytochrome c from mitochondria occurs before or after loss of $\Delta\Psi m$. This information is important because it will reveal the death pathway responsible for removal of hypertrophic chondrocytes from the cartilage-bone interface, offering new therapeutic targets (such as MAC pharmacological inhibitors) for growth anomalies resulting from alterations of that process.

In addition, chondrocytes have been shown to play a crucial role in fracture repair and distraction osteogenesis. In the near future, it might be possible to accelerate the bone healing process by manipulating chondrocyte removal and osseous deposition. Finally, articular chondrocytes can behave just like hypertrophic chondrocytes during osteoarthritis by mineralizing the cartilage matrix and dying inappropriately, converting a permanent cartilage into a transient one. Therefore, the study of growth plate chondrocyte death may provide insight into new therapeutic approaches for this widespread and debilitating problem.

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FIGURES Return to TOC

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Figure 1. Model of apoptotic pathway involved in inorganic phosphate(Pi)–induced death of chondrocytes. Pi treatment leads to a loss of mitochondrial membrane potential ($\Delta\Psi m$), provoking the release of cytochrome c and other proteins into the cytosol, activating caspase 3, and starting a cascade of protease activity that ultimately leads to endonuclease activation, DNA degradation, and cell death

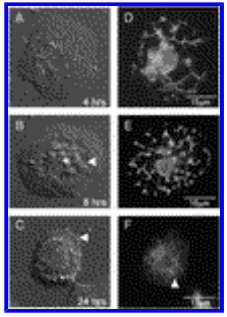

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Figure 2. Functional and morphological alterations in chondrocytes undergoing apoptosis. Chondrocytes were treated with 5 mM inorganic phosphate (Pi) for 4 hours (A and D), 8 hours (B and E), and 24 hours (C and F). Differential interference contrast (A-C) and fluorescence (D-E) microscopy images of the same cells are shown. Functional mitochondria were labeled with TMRE (red fluorescence in D-E), and chondrocyte DNA was labeled with Hoechst dye 33342 (blue fluorescence in D-E). Note the presence of numerous vesicles in the cytoplasm (arrowhead in B) of a dying chondrocyte. At 24 hours after initiation of Pi treatment, the chondrocyte shows obvious signs of apoptosis: loss of attachment, shrinkage, blebbing (arrowhead in C), and apoptotic bodies containing DNA (arrowheads in F)

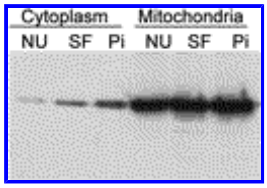

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Figure 3. Inorganic phosphate (Pi) causes release of cytochrome c from mitochondria. Western Blot analysis was performed on chondrocytes cultured for 24 hours in the presence of 10% NUSERUM (NU), in serum-free media (SF), and in serum-free media supplemented with 5 mM phosphate (Pi). After cell fractionation, proteins from the cytoplasmic and mitochondrial fractions were separated electrophoretically, transferred onto a nitrocellulose membrane, and immunostained with antibody against cytochrome c. The same amount of protein was loaded into each column

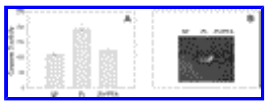

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Figure 4. Treatment with inorganic phosphate (Pi) increases caspase 3 and endonuclease activities in apoptotic chondrocytes. Chondrocytes were cultured for 24 hours in serum-free media (SF) and serum-free media supplemented with 5 mM Pi, in the presence or absence of 5 mM phosphonoformic acid (PFA). Caspase activity was evaluated by measuring fluorescence in the presence of the substrate Z-DEVD-AFC (A). Endonuclease activity was identified by zymographic analysis (B) as clear spots on the dark background

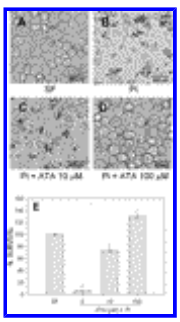

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Figure 5. Endonuclease inhibitor prevents inorganic phosphate (Pi)– induced chondrocyte apoptosis. Chondrocytes were cultured for 24 hours in serum-free media (SF) (A) and in serum-free media supplemented with 5 mM Pi (B). Some chondrocytes were treated with 5 mM of Pi in the presence of different concentrations of ATA (10 μM, 100 μM) (C-D). After 24 hours, cells were observed by regular light microscopy and photographed. Chondrocytes survival under the same experimental conditions was estimated by MTT assay (E) with results presented as percentage of control (SF). Values shown represent three separate experiments. *Significantly different from control ($P < .05$)

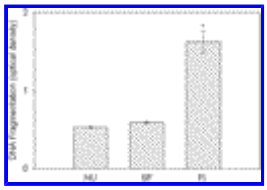

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Figure 6. Inorganic phosphate (Pi) induces DNA fragmentation in chondrocytes. Chondrocytes were cultured for 24 hours in the presence of 10% NUSERUM (NU), in serum-free media (SF), and in serum-free media supplemented with 5 mM Pi. At the end of the cultured period, cells were lysed, and then histone-complexed DNA fragments were detected with a colorimetric ELISA assay. *Significantly different from NU and SF ($P < .05$)

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