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The Role of Apoptosis in Mineralizing Murine Versus Avian Micromass Culture Systems

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ABSTRACT

Chondrocyte apoptosis is thought to be an important step in the calcification of cartilage in vivo; however, there are conflicting reports as to whether or not this apoptosis is a necessary precursor to mineralization. The goal of this study was to determine whether or not apoptosis is necessary for mineralization in an in vitro murine micromass model of endochondral ossification. C3H10T1/2 murine mesenchymal stem cells were plated in micromass culture in the presence of 4 mM inorganic phosphate with the addition of the apoptogens, camptothecin, or staurosporine, to induce apoptosis. The rate and total accumulation of mineralization was measured with ^{45}Ca uptake. In these studies, both apoptogens increased the rate of mineralization, with staurosporine increasing ^{45}Ca accumulation by about 2.5 times that of controls and camptothecin increasing total amounts of mineralization about 1.5 times that of controls. Inhibiting cell apoptosis with the caspase inhibitor, ZVAD-fmk, to prevent apoptosis, caused slower rates of ^{45}Ca uptake; however, total amounts of ^{45}Ca accumulation reached the same values by day 30 of culture. FTIR data showed mineralization in all samples treated with 4 mM inorganic phosphate, with the highest mineral to matrix ratios in the camptothecin treated samples. J. Cell. Biochem. 111: 653–658, 2010.

KEY WORDS: APOPTOSIS; MINERALIZATION; MICROMASS CULTURES

Endochondral ossification occurs through a series of events that are not yet completely understood. There is evidence that terminally differentiated, hypertrophic chondrocyte apoptosis is one step in the process that leads to cartilage calcification and growth of long bones [Gibson et al., 1995; Roach et al., 1995; Roach, 1997; Gibson, 1998; Adams and Shapiro, 2002]. There are, however, conflicting reports as to whether or not apoptosis is an essential step in the mineralization process [Gibson, 1998; Felisbino and Carvalho, 2001; Kirsch et al., 2003; Magne et al., 2003; Mansfield et al., 2003].

We previously reported that in primary chick limb bud micromass cultures, apoptosis is not necessary for mineralization to occur, and that the induction of apoptosis did not increase mineralization [Pourmand et al., 2007]. These studies contrast with data from other groups using the murine cell line, ATDC5, which undergoes chondrogenic differentiation with the addition of insulin. Supplementation with exogenous inorganic phosphate triggers apoptotic cell death leading to mineralization in these cultures [Magne et al., 2003]. These studies suggest that apoptosis mediated mineralization may be a species dependent phenomena, and that while there are similarities between avian and mammalian endochondral ossification, they may not be identical processes. These data also imply that the signals that the chondrocytes require to go through the process from differentiation to maturation and hypertrophy may be dependent on cell source.

Micromass spot cultures, or high-density cultures, have previously been used as a model of endochondral ossification [Boskey et al., 1992a, 1996a,b; Mello and Tuan, 1999; Mello and Tuan, 2006; Pourmand et al., 2007]. The high density of cells and close cell-to-cell contacts create an environment that promotes and supports chondrocyte differentiation [Denker et al., 1999; Haas and Tuan, 1999; Delise et al., 2000]. These micromass cultures produce a mineralized matrix with the addition of exogenous inorganic phosphate, and provide a controlled environment in which the effects of apoptosis on calcification can be quantified [Boskey et al., 1991, 1992a,b, 1996a,b, 1997, 2002, 2008].

The induction of apoptosis is an event that can be triggered by both internal and external cellular pathways [Mirkes, 2002]. Activation of intracellular signaling involving caspase cascades has been implicated as the primary pathway of apoptosis [Cohen, 1997; Chowdhury et al., 2008; Yi and Yuan, 2009]. Both staurosporine and camptothecin have been used as effective external apoptosis inducers (apoptogens) via activation of one or

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more of these caspase pathways. To inhibit apoptosis, Z-Val-Ala-Asp(O-Me)-fluoromethylketone (ZVAD-fmk), is an effective agent that prevents staurosporine and camptothecin induced apoptosis in chondrocytes and other cell types [Yue et al., 1998; Lee et al., 2000; Ceccatelli et al., 2004].

The objective of this study was to compare these apoptotic pathways with the in vitro micromass model of endochondral ossification and to clarify the role of apoptosis in mammalian versus avian mineralization. It was hypothesized that apoptosis is a significant event in mineralization of the micromass cultures of the murine cell line C3H10T1/2, and that the induction of apoptosis will lead to increased mineralization in contrast to the previously reported data from avian cultures. Moreover, this study aimed to determine whether or there are species specific or reagent specific differences in the avian and murine systems.

MATERIALS AND METHODS

CHICK CULTURES

Chick limb-buds were obtained at stage 21–24 [Hamburger and Hamilton, 1951] from white Leg Horn chick embryos. Mesenchymal cells released by digestion in trypsin–EDTA (GIBCO) were separated from debris by passage through a 20 μm Nitex membrane, counted with a hemocytometer, checked for viability by trypan blue dye exclusion, and pelleted in the cold at 1,100 rpm. Cells, resuspended in DMEM with 100 units/ml of penicillin, 100 μg/ml of streptomycin base, and 0.25 mg/ml amphotericin B, (1% antibiotics/antimycotics) were plated using the micro-mass technique [Ahrens et al., 1977] at a density of 0.75×10^6 cells per 20 μl drop in 35 mm × 10 mm Falcon dishes, allowed to attach for 1 h in a humidified atmosphere of 5% CO_2 at 37°C, and then flooded with DMEM (GIBCO 80-0303A); containing 1 g/L glucose and supplemented with 50 units/ml penicillin and 25 μg/ml streptomycin, and 10% fetal bovine serum (FBS) (Atlanta Biologics). From day 2 onward, vitamin C (25 μg/ml) was added along with glutamine (0.3 mg/ml) and for all mineralizing cultures the total phosphate concentration (Pi) was adjusted to 4.0 mM with potassium acid phosphate. Control non-mineralizing cultures received no phosphate addition, and were ~1.0 mM in Pi. Cultures were incubated at 37° with 95% air, 5% CO_2 with media changed three times per week. Cultures were treated with 11.5 μM camptothecin (Camp) in DMSO beginning on day 7 and continuing through the entire culture period to induce apoptosis in some dishes as previously described. Control dishes were treated with DMSO only.

MURINE CELLS

C3H10T1/2 cells (ATCC, CCL-226) were expanded in DMEM (Invitrogen), 10% FBS (Invitrogen), and 1% antibiotics/antimycotics. Cells were passaged two times per week (0.05% trypsin, Invitrogen) at about 85% confluency. Passage 7–12 cells were used for experiments.

For micromass cultures cells were plated at a density of 100,000 cells per 10 μl spot in the center of 35 mm tissue culture dishes and allowed to attach for 2 h prior to flooding with media as detailed elsewhere [Roy et al., 2010]. Cultures were maintained in DMEM with 1% FBS, 6.25 μg/ml of recombinant insulin, 6.25 μg/ml human transferrin, and 6.25 ng/ml selenious acid and 1% antibiotics/antimycotics in an incubator at 37°C with 5% CO_2. From day 2 onward micromass cultures received 1.3 mM $CaCl_2$, 25 μg/ml of ascorbic acid, and 1% L-glutamine. Also beginning on day 2 of culture, 4 mM inorganic phosphate (4P) was added to media for mineralizing cultures while control cultures remained with 1 mM inorganic phosphate (1P). On day 7 of micromass cultures, 40 nM staurosporine (Stauro) or 11.5 μM camptothecin (Camp) in DMSO was added to the media and for every media change thereafter to induce apoptosis in some dishes as previously described [Pourmand et al., 2007]. Control dishes were treated with the carrier, DMSO. In experiments to inhibit apoptosis, the pan-caspase inhibitor, ZVAD-fmk, was given to cultures also from day 7 onward.

FLOW CYTOMETRY

Flow cytometry with FITC labeled annexin V and 7-AAD (BD Biosciences) was used to confirm apoptosis and cell death as previously described [Pourmand et al., 2007]. In summary, the media from six samples were removed and combined with the 1 ml of trypsin (0.25%, Invitrogen) that was added to cultures and incubated for 5 min at 37°C. The media and trypsin–cell mixture was centrifuged at 1,100 rpm for 8 min and the supernatant was discarded. The cell pellet was then mixed with 200 μl of binding buffer (BD Biosciences). Cells were stained with annexin V-FITC to determine apoptosis and 7AAD to determine death for 15 min at room temperature and then placed on ice before flow cytometry. Samples were analyzed with FloJo software and were gated for cell death or apoptosis based on unstained and single stain controls.

CALCIUM UPTAKE

^{45}Ca was added to the media also starting on day 7 and samples analyzed as previously described for mineral accretion [Pourmand et al., 2007]. ^{45}Ca scintillation was normalized to day 28 mineralizing DMSO cultures (4P) after subtracting control (1P) DMSO values. For each group there was a sample number of 5–7 from three independent experiments.

FOURIER TRANSFORM INFRARED SPECTROSCOPY

For FTIR studies cultures were thoroughly washed with 100% ethanol and allowed to air-dry overnight. Freshly dried (120°C, 24 h) KBr (200 mg) was mixed with the air-dried samples in the tissue culture plates and pellets were made for spectroscopic analysis. For FTIR studies 4–6 samples were pooled for each spectrum and values were averaged from independent studies. Spectra were first baselined and the mineral to matrix ratio was determined as the area under the phosphate peak (900–1,200 cm^{-1}) divided by the area under the amide I peak (1,585–1,720 cm^{-1}). Collagen maturity was defined as the ratio of reducible to non-reducible crosslinks (1,660/1,690) [Paschalis et al., 2003] and hydroxyapatite crystallinity [Pleshko et al., 1991] was determined from the ratio of the peak intensity at 1,030/1,020.

RESULTS

Flow cytometry was used to confirm the effectiveness of the apoptogens at selected time points. With camptothecin, in the C3H10T1/2 cultures, there was significantly more apoptosis (~21%) than with apoptogen-free (DMSO-treated) control cultures (~1%) at day 9 (2 days after treatment with camptothecin). Similarly, in the chick cultures treated with camptothecin, there were significantly higher amounts of apoptosis (~21%) at day 9 than controls treated with DMSO (~8%) (Fig. 1A). In chick cultures, camptothecin induced apoptosis earlier than in murine cultures (Fig. 1A), and there were large amounts of late apoptotic cells (~13%). On day 9 staurosporine treated murine cultures had the highest percentage of early apoptotic cells (~25%) (Fig. 1A). This is similar to rates of apoptosis previously reported for similar studies in chick micromass cultures [Pourmand et al., 2007]. By day 12, in the murine cells, there were higher amounts of apoptosis in all cultures, including controls (Fig. 1B). The murine cultures treated with camptothecin and staurosporine both had a large percentage of apoptotic cells (~30% and ~45%, respectively) (Fig. 1B). Control cultures at day 12 also had a percentage of cells undergoing apoptosis (~17%). By day 12 there were also higher numbers of dead murine cells in all conditions. ZVAD-fmk treated cultures showed significantly fewer cells undergoing apoptosis and cell death than all other conditions including control cultures (~5%) (Fig. 1B).

Based on earlier studies apoptosis in chick micromass cultures [Pourmand et al., 2007], spots were treated with an apoptogen beginning at day 7 and throughout the rest of the culture period. When the apoptogen, camptothecin, was given to 4P chick cultures at day 7, cultures failed to mineralize and ^{45}Ca values remained at levels similar to the 1P controls (Fig. 2A). Chick cultures treated with camptothecin and 4P after the start of mineralization, at day 14, mineralized normally, with no differences compared to the 4P control (DMSO treated) cultures (Data not shown).

All mineralizing (4P) murine cultures showed increased ^{45}Ca uptake, while 1 mM cultures did not show significant mineralization. Both staurosporine and camptothecin treated mineralizing cultures showed higher amounts of ^{45}Ca uptake compared to control mineralizing (4P DMSO) cultures, with staurosporine treated cultures reaching a plateau 2.5 times that of the controls (Fig. 2B).

ZVAD-fmk is a pan-caspase inhibitor shown to block the pathways that lead to chondrocyte cell death by apoptosis [Garcia-Calvo et al., 1998]. Mineralizing murine micromass cultures treated with ZVAD-fmk, had slower rates of ^{45}Ca uptake compared to

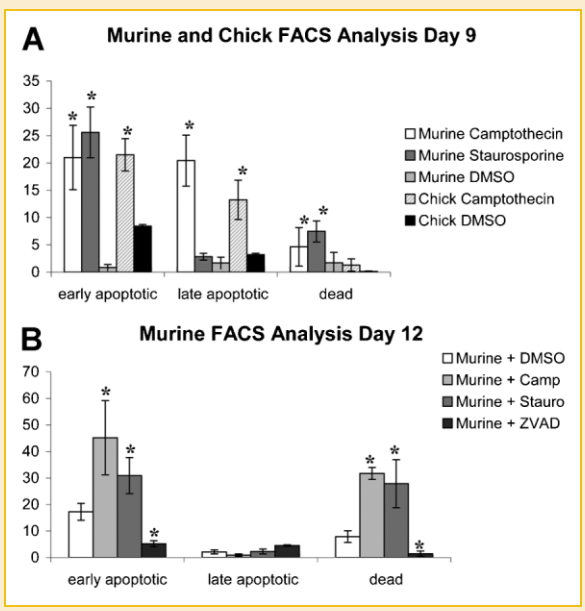

Fig. 1. A: FACS analysis of murine and chick cultures at day 9. Both camptothecin and staurosporine induced apoptosis in murine cultures. There were more late apoptotic (double-labeled) cells with camptothecin treatment than staurosporine treatment. Chick cultures treated with camptothecin showed a similar profile for apoptosis. $^{*}P < 0.05$ versus control cultures. B: FACS analysis of murine cultures at day 12. At day 12 (5 days after start of apoptogen treatment) only murine cultures were analyzed. There were more cells undergoing apoptosis with staurosporine treatment and camptothecin treatment compared to controls. There were also more cells undergoing apoptosis in control cultures on day 12 than day 9, likely due to the start of mineralization. ZVAD-fmk treated cultures showed significantly less apoptosis than control cultures. There were significantly more dead cells with apoptogen treatment than control cells, and ZVAD treatment of the murine cultures resulted in a lower amount of dead cells than all other conditions, including control cultures. There were no differences in the amounts of late apoptotic cells by day 12. $^{*}P < 0.05$ versus control cultures.

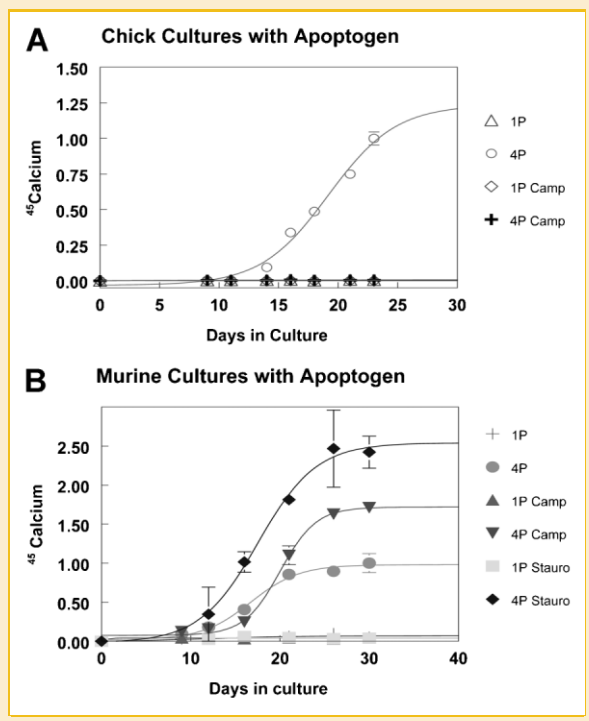

Fig. 2. A: ^{45}Ca uptake in chick cultures treated with apoptogen. The treatment of chick cultures with camptothecin completely inhibited mineralization, and ^{45}Ca uptake values were similar to non-mineralizing control (1P) cultures. B: ^{45}Ca uptake in murine cultures treated with apoptogen. Both staurosporine and camptothecin caused an increase in the rate and extent of ^{45}Ca uptake in murine cultures. Treatment with staurosporine resulted in the highest amount of mineral accretion, with values about 2.5 times that of control mineralizing (4P) cultures.

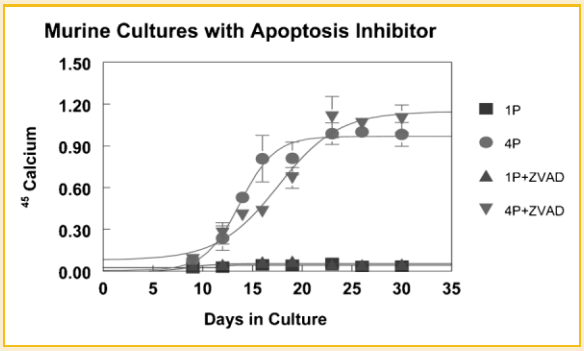

Fig. 3. ^{45}Ca uptake in murine cultures treated with an apoptosis inhibitor. Treatment of murine cultures with ZVAD-fmk resulted in a slower rate of mineralization than control mineralizing (4P) cultures, but the extent of mineralization was not significantly different than controls (4P).

control. The total amount of mineralization, however, reached control levels (4P + DMSO) by day 23 and the plateau levels of ^{45}Ca uptake were not significantly different (Fig. 3).

FTIR spectroscopy was performed on murine day 28 samples as apoptogen treated samples often detached, making analysis at later time points difficult. The FTIR spectroscopy shows some mineralization at day 28 in all groups given 4P (Fig. 4). In the staurosporine and camptothecin treated samples, there were higher mineral to matrix ratios compared to control 4P cultures (camptothecin 1.91 ± 0.01, staurosporine 1.40 ± 0.16, control DMSO 0.51 ± 0.11, ZVAD-fmk 0.49 ± 0.03), supporting the ^{45}Ca uptake data, and suggesting that increases in apoptosis parallel the extent of mineralization in the murine cultures. There were, however, changes in the amide II peak with the addition of both apoptogens, but not with the apoptosis inhibitor, possibly indicating a deficiency or defect in the matrix in these cultures.

DISCUSSION

The goal of this study was to determine whether or not apoptosis is a prerequisite to chondrocyte calcification in a mammalian system, or

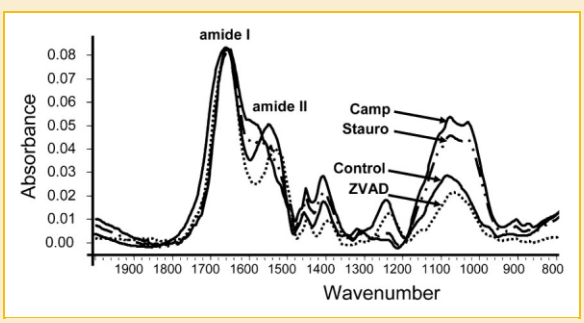

Fig. 4. FTIR spectroscopy of murine cultures. The FTIR spectroscopy showed more area under the mineral peaks in cultures treated with an apoptogen than control and ZVAD-fmk treated cultures. The shape of the amide ii peak in cultures treated with both apoptogens was different than control and ZVAD-fmk treated cultures, indicating differences in the collagen produced.

whether differences previously reported were due to the specific apoptogens used. In similar studies with primary chick limb-bud micromass cultures, apoptosis was not required for mineralization [Pourmand et al., 2007]. In the chick, blocking apoptosis had no significant effect on measured mineralization parameters and inducing apoptosis with two different agents camptothecin, in the present study, and staurosporine, in a previous study, caused decreases in micromass matrix mineralization [Pourmand et al., 2007]. This is contrary to some reports of what is hypothesized to occur in vertebrates in vivo [Bronckers et al., 1996; Zenmyo et al., 1996; Aizawa et al., 1997; Amling et al., 1997] and even studies of avian bone growth [Gibson et al., 1995; Hatori et al., 1995; Ohyama et al., 1997; Gibson, 1998]. The present study employed a murine cell line to mimic endochondral ossification, in vitro, and to determine whether there are species-specific roles of apoptosis in cartilage mineralization.

We found increases in mineralization of murine micromass cultures when treated with two different apoptogens. Based on previous studies and preliminary data, these agents were given prior to the start of mineralization [Pourmand et al., 2007], and were shown by fluorescence activated cell sorting (FACS) analysis to increase apoptosis. While staurosporine treated cultures showed the highest total amounts of calcium uptake, FTIR showed the highest mineral to matrix ratios for camptothecin treated cultures. These data along with the FACS analysis suggest that because the camptothecin treated cultures underwent apoptosis earlier, they possibly may have synthesized less extracellular matrix. The staurosporine treated cultures may have had more time to deposit the extracellular matrix, resulting in the lower mineral to matrix values observed by FTIR. These data also confirm that the collagen or interactions of matrix proteins made by the cells with collagen is crucial to proper mineralization and crystallinity [Kuznetsova et al., 2004; Roschger et al., 2008; Xu et al., 2008].

The changes in the amide II region in the FTIR spectra of apoptogen-treated cultures may indicate changes in the matrix produced by apoptotic cells. It is possible that the early treatment with apoptogens does not give the cells time to make the correct post-translational modifications to the collagen. When given an apoptogen, there also may be little time for the chondrocytes to mature and hypertrophy causing deficiencies in matrix formation before cell death.

The differences in the increase in the total extent of mineralization, seen in the murine cultures, between the two apoptogens as measured by ^{45}Ca could be due to the different caspase cascades involved. Staurosporine works through caspases 3 and 9 [Yue et al., 1998; Shiraishi et al., 2001; Ceccatelli et al., 2004], while camptothecin induces apoptosis through caspase 2 and 3 [Stefanis et al., 1999]. In addition, camptothecin has been shown to interact with DNA topoisomerase I causing an intrinsic cascade of cell apoptotic death. It is possible that, as shown by our flow cytometry data, the different patterns of cell apoptosis and cell death led to slightly different responses for these apoptogens. This may be due to the differences in time of apoptosis in relation to the start of mineralization. It is important to note that camptothecin also induced apoptosis in the chick cultures in a similar manner to the chick cultures treated with staurosporine, and in both cases

the chick cultures did not mineralize properly [Pourmand et al., 2007].

Both apoptogens also showed increases in the rate of mineralization as measured by ^{45}Ca when given to murine cultures. This indicates that when increasing the amount and time course for apoptosis, mineralization occurs faster in the C3H10T1/2 cultures. This idea was also supported by the ZVAD-fmk studies. Inhibition of apoptosis through interference with the caspase cascade slowed the rate of mineralization in ZVAD-fmk treated murine micromass cultures. Control cultures also exhibited a certain amount of apoptosis and showed rates of mineralization in between the ZVAD-fmk treated and apoptogen treated cultures, indicating that apoptosis does play a role in mineralization in the murine system.

There were changes in the rate of mineralization with inhibition of apoptosis, but not in the total extent of mineralization as shown by the calcium uptake curves for ZVAD-fmk treated murine micromass cultures. These data indicate that while apoptosis may play a role in endochondral ossification, it is not a required step in mineralization. There are reports that vascular invasion of cartilage at the growth plate is linked to apoptosis, and furthermore that inhibition of angiogenesis results in an accumulation of hypertrophic chondrocytes in vivo [Gerber and Ferrara, 2000]. Our in vitro results suggest that while induction of apoptosis may create an environment or one pathway to calcification, this is not the primary or only method of calcification of chondrocytic cells. Studies show that osteogenic differentiation, mineralization, and apoptosis and can be regulated by phosphate and calcium concentrations [Mansfield et al., 2001; Magne et al., 2003; Liu et al., 2009]. It is possible that these are cell-type specific and that apoptosis is not the major factor in normal endochondral ossification. There are data that support this theory, as in fractures, the inhibition of apoptosis has been shown to increase healing [Garcia et al., 2010].

When added to the chick cultures, camptothecin behaved like staurosporine and did not increase the extent of calcification, suggesting this was not a drug-specific effect. This is supported by data from the living mouse where treatment with camptothecin enhanced mineralization [Li et al., 2005]. Based on the data in this study as well as others, it is likely that the role of apoptosis in mineralization is species specific.

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