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Intracellular Zinc Fluxes Associated With Apoptosis in Growth Plate Chondrocytes

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Abstract Matrix vesicles released by epiphyseal growth plate chondrocytes are known to contain a significant quantity of labile Zn^{2+}. Zonal analysis of chicken metatarsal bones showed that the resting/proliferative region of the growth plate contained high levels of Zn^{2+} with significantly lower levels in the hypertrophic cartilage suggesting a loss of cellular Zn^{2+} as the chondrocytes mature. Intracellular labile Zn^{2+} was measured in primary cultures of growth plate chondrocytes by assay with the fluorescent Zn-chelator toluenesulfonamidoquinoline (TSQ) and imaged by multi-photon laser scanning microscopy (MPLSM) with the TSQ derivative zinquin. Short-term exposure to Zn^{2+}, both in the presence and absence of pyrithione resulted in significant increases in cytosolic Zn^{2+}. Treatment with the membrane-permeant Zn^{2+} chelator TPEN rapidly reduced the levels of labile Zn^{2+} and triggered apoptosis. Cytosolic Zn^{2+} levels were significantly reduced following 24-h incubations with known inducers of chondrocyte apoptosis. The loss of intracellular Zn^{2+} was accompanied by a significant reduction in the cytosolic metal-binding protein metallothionein. Examination of Zn^{2+}-treated cells with MPLSM showed uniformly higher zinquin fluorescence. Treatment of Zn^{2+}-loaded cells with TPEN quenched zinquin fluorescence confirming that the observed fluorescence in chondrocytes is due to the presence of intracellular Zn^{2+}. A dose-dependent increase in zinquin fluorescence was observed in cells treated with a range of Zn^{2+} concentrations. Short-term treatment of cultured chondrocytes with apoptosis-inducing chemicals resulted in transient increases in intracellular labile Zn^{2+}. These results indicate that Zn^{2+} is mobilized from intracellular binding sites in the early stages of chondrocyte apoptosis and is subsequently lost from the cells. The early mobilization of Zn^{2+} pro vides a mechanism for its movement to matrix vesicles and the extracellular matrix. J. Cell. Biochem. 88: 954–969, 2003.

Key words: cartilage; multi-photon laser scanning microscopy; programmed cell death; metallothionein

Zinc is a normal constituent of vertebrate bones and teeth and is essential to the formation of skeletal tissues. It has long been known that Zn^{2+} deficiencies can result in severe skeletal disorders [Guggenheim and Gaster, 1972]. Early histochemical studies showed a co-localization of Zn^{2+} and sudanophilic material in the extracellular matrix of epiphyseal growth plate cartilage [Asling and Hurley, 1963.]. The sudanophilic reaction was found to be due to the presence of extracellular matrix vesicles (MV) [Anderson, 1969] which are now known to be the initial sites of mineral deposition in epiphyseal cartilage, dentine, and other mineralizing tissues [Wuthier, 1988]. More recently, we found that MV contain unusually high levels of Zn^{2+}, and that this ion regulates the onset of the mineralization process [Sauer et al., 1989]. It has also been shown that supplementation of serum-free cultures of growth plate cartilage chondrocytes with low levels of zinc stimulates cell proliferation and growth [Litchfield et al., 1998; Rodriguez and Rosselet, 2001].

Endochondral bone formation represents the primary mechanism of vertebrate skeletal development. Progressive growth of the epiphyseal

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growth plate is the means by which longitudinal bone elongation occurs [Hunziker, 1994]. As originally suggested over 20 years ago [Kardos and Hubbard, 1982], it is now widely recognized that growth plate chondrocytes undergo apoptosis at the terminal stage of their normal developmental process [Gibson et al., 1995; Hatori et al., 1995; Zenmyo et al., 1996; Ambling et al., 1997; Roach, 1997]. Nutritional studies have generally shown that zinc deficiency results in increased rates of apoptosis in a variety of tissues including growth plate cartilage [Elmes, 1977; Chai et al., 1999; Wang et al., 2002]. A large number of laboratory studies indicate a role for Zn^{2+} ions in the regulation of apoptosis [Sunderman, 1995].

Toluenesulfonamidoquinoline (TSQ) is a commercially available quinoline compound, which forms UV-fluorescent complexes with Zn^{2+} and has been used as a histochemical stain for Zn^{2+} in tissue sections [Frederickson et al., 1987]. Recently, an esterified derivative of TSQ, designated Zinquin, has been produced which allows the fluorophore to be taken up by live cells. Once inside the cell, the ester group is cleaved by intracellular esterases leaving the negatively charged probe trapped within the cell [Zalewski et al., 1993]. Zinquin is highly specific for Zn^{2+} and is sensitive to nanomolar concentrations of free Zn^{2+}. The Zn^{2+}-dependent fluorescence is unaffected by over thousand-fold molar excesses of physiologically relevant cations such as Mg^{2+} and Ca^{2+}. Zinquin has been used to study zinc fluxes in thymocytes [Zalewski et al., 1993] and lymphoma cells [Smith et al., 2002], the relationship between glycolysis and Zn^{2+} metabolism in hepatocytes [Brand and Kleineke, 1996], and the vesicular accumulation of Zn^{2+} in neurons [Palmiter et al., 1996]. Studies with zinquin have shown that apoptosis is accompanied by transient increases in cytosolic Zn^{2+} [Zalewski et al., 1994; Smith et al., 2002]. It is not clear whether these Zn^{2+} fluxes are critical to the continuation of the apoptotic cascade or are a secondary consequence of this process.

We previously established an avian chondrocyte culture system that mimics the terminal differentiation and calcification process of growth plate chondrocytes [Wu et al., 1995]. Using this culture system we established the importance of Zn^{2+} in chondrocyte growth and development [Litchfield et al., 1998] and observed distinct phenotypic differences in the expression of metallothionein, the principle intracellular Zn^{2+}-binding protein [Sauer et al., 1998], that suggest a role for metallothionein and Zn^{2+} in chondrocyte differentiation and apoptosis. In the present study we used wet chemical analysis to determine the zinc concentrations in different regions of the growth plate. Because of the growing realization of the importance of zinc in the regulation of apoptosis, we used TSQ and zinquin to determine if the distribution of Zn^{2+} in chondrocytes changes during chemically induced apoptosis. We also sought to determine whether the mobilization of Zn^{2+} ions from intracellular sites during apoptosis could explain the high levels of labile Zn^{2+} found in MV. The results indicate a role for Zn^{2+} in the regulation of apoptosis in growth plate chondrocytes.

MATERIALS AND METHODS

Materials

Broiler strain chickens (6–8 weeks old), to provide growth plate tissue for chemical analysis and cell isolation, were obtained from Columbia Farms (Columbia, SC). Ultrapure nitric acid for chemical analysis was purchased from GFS Chemicals (Columbus, OH). Fetal bovine serum was from Atlanta Biologicals (Atlanta, GA), and all cell culture media and buffers were from Sigma Chemical (St. Louis, MO). Zinquin ester and zinquin free acid was purchased from Luminis Pty. Ltd. (Adelaide, South Australia). *N*-(6-methoxy-8-quinolyl)-*p*-toluenesulfonamide (TSQ) was purchased from Molecular Probes (Eugene, OR). The Roche Molecular Biochemicals In Situ Cell Death Detection Kit, Fluorescein was from Boehringer Manheim (Indianapolis, IN). All other chemical reagents used in this study were purchased from Sigma Chemical. All glassware and disposable plasticware (Corning) were purchased from Fisher Scientific (Atlanta, GA).

Zinc and Calcium Analysis

Thin slices of tissue from the growth plates of 6–8 week-old chicken metatarsal bones were collected under ice-cold heptane for chemical analysis. The epiphyseal growth plate was exposed by removing the overlying articular cartilage and three distinct regions of the growth plate were separated by visual inspection of the tissue during dissection. These were designated as resting/proliferative zone (upper

2 mm), hypertrophic zone (2–6 mm depth), and calcifying zone (4–10 mm depth). A portion of the bone tissue underlying each growth plate was also processed for analysis. The dissected tissues from 28 metatarsal bones were pooled, lyophilized, and ground into a fine powder with an acid-washed mortar and pestle. Five aliquots of the powdered tissue were weighed and digested in 4 ml of ultra pure nitric acid (GFS Chemicals, Columbus, OH) heated to a gentle reflux at approximately 80°C. The tissue digests were diluted to known volumes with glass-distilled de-ionized water and analyzed for calcium and zinc on a Perkin Elmer 403 Atomic Absorption Spectrophotometer using an air/acetylene flame. Analytical results were authenticated by performing identical analyses on U. S. National Bureau of Standards certified bovine liver (Standard Reference Material 1577a). The levels of zinc and calcium determined for the liver samples were within 5% of the certified values. All glassware used in these analyses were acid-washed prior to use to eliminate metal contaminants.

Cell Culture

Chondrocytes were isolated from small blocks of epiphyseal growth plate tissue from tibiae of 6- to 8-week old hybrid chickens by trypsin and collagenase digestion as described previously [Wu et al., 1995]. Cells were plated at a density of 4.5×10^5 cells/35-mm dish and cultured in 2 ml Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere. Cells used for zinquin fluorescence and confocal visualization of apoptosis were plated in 4-chambered cover glasses (Nunc) at a density of 1.1×10^5 cells/well and cultured in DMEM/10% FBS. The culture medium was changed every 3 days and ascorbate was provided from day 3 onwards at a concentration of 50 μg/ml. All experiments were performed on 24- to 28-day old cultures unless otherwise indicated. This age of culture corresponds developmentally with the hypertrophic phenotype as we have shown previously [Wu et al., 1995; Sauer et al., 1998]. To induce apoptosis in cultured cells, dexamethasone 0.1–1 μM, sodium nitroprusside (SNP) 50 μM, or antibiotic A-23187 (10 nM) were added to normal culture media in a minimal volume (<10 μl) from sterile stock solutions to achieve the indicated concentrations. To experimentally alter intracellular levels of zinc, cells were treated with *N*,*N*,*N*′,*N*′-tetrakis (2-pyridyl-methyl)ethylenediamine (TPEN), zinc chloride, or zinc chloride plus 1-hydroxypyridine-2-thione (pyrithione) for 1 h prior to cell harvesting. These reagents were added from stock solutions directly to the culture medium to achieve a final concentration of 100 μM. At the conclusion of each experiment, the culture medium was removed and the cells were washed twice with ice-cold phosphate buffered saline (PBS). Cells from each dish were harvested into 1.5-ml ice-cold 10 mM Tris (pH 7.4) containing 100 mg/L digitonin and frozen at −20°C for storage prior to analysis. For analysis, the frozen samples were thawed and subjected to sonication to ensure complete lysis of the cells. Cell debris and matrix material was removed from the cell sonicate by centrifugation at 28,000*g* for 20 min in a refrigerated microcentrifuge. Each experiment was repeated two or three times with different primary cultures.

Analysis of Cell Lysates

Prior to centrifugation, the DNA content of the cell sonicate were determined by Hoechst 33258 dye binding (BioRad, Hercules, CA). After centrifugation, cytosolic protein in the cell lysate was determined by the Bradford assay [Bollag and Edelstein, 1991]. Aliquots (0.2 ml) of the cell lysate were taken for quantitation of metallothionein by the Cd-hemoglobin affinity assay [Eaton and Cherian, 1991] and for zinc by a fluorescence assay. For fluorescent analysis of zinc, 0.4 ml aliquots of the cell lysate was combined with 0.1 ml of 10 mM Tris (pH 7.4) containing 50 μM TSQ. TSQ is selective for Zn^{2+} in the presence of physiological concentrations of other divalent cations. TSQ-Zn^{2+} fluorescence was measured on a Shimadzu RF-5301PC spectrofluorophotometer using an excitation wavelength of 375 nm and emission at 485 nm. The fluorescence standard curve for Zn^{2+} produced by adding the TSQ reagent buffer to solutions containing known concentrations of $ZnCl_2$ was linear over a range of 0.02–5 μM.

Confocal and Multiphoton Microscopy

Intracellular labile Zn^{2+} was visualized by zinquin fluorescence using multi-photon laser scanning microscopy (MPLSM). Chondrocytes grown in cover glass chambers were washed twice with Hank's Balanced Salts Solution (HBSS) and incubated for 60 min at 37°C in

HBSS containing 15 μM zinquin ester. Following incubation with zinquin, the cultures were rinsed three times with HBSS and held at room temperature in fresh HBSS for 1–2 h during which zinquin fluorescence was examined. As a positive control some cells were loaded with Zn^{2+} by treating cells for 1 h with the Zn^{2+} ionophore pyrithione [Zalewski et al., 1991] in the presence of 100 μM Zn^{2+}. To confirm that Zinquin fluorescence was due to intracellular Zn^{2+}, some cells were treated with the membrane-permeant Zn^{2+}-chelator TPEN [Sensi et al., 1997]. Zinquin fluorescence was visualized on a Bio-Rad 1024 MP confocal laser microscope using a tunable Ti:Sapphire laser operated at 770-nm excitation wavelength. In preliminary experiments with the Ti:Sapphire laser, 770 nm was empirically determined to give optimal two-photon excitation of zinquin and corresponds to the maximum florescence observed at 365 nm with single photon UV laser excitation. Emitted light images were collected using a 460-nm cut-off (lower limit) filter. The 460-nm filter eliminated a small amount of auto-fluorescence arising from collagen in the extracellular matrix of these cultures. In order to permit comparison of Zinquin images between treatments, instrumental settings were held constant during each analysis. To determine the affinity of zinquin for Zn^{2+} under intracellular conditions, Hill plot analysis was conducted using zinquin acid in physiological buffer designed to mimic intracellular conditions containing 120 mM KCl; 30 mM NaCl; 1 mM $MgSO_4$; 10 mM MOPS; and 1 μg/ml bovine serum albumin. Fluorescent intensities were determined with a Shimadzu RF-3501 PC Spectrofluorophotometer for a series of added zinc concentrations ranging from 1 nM to 0.1 mM, $[Zn^{2+}] = 0$, and at saturating $[Zn^{2+}]$ using excitation at 365 nm and emission at 490 nm.

In some studies, zinquin was used to estimate the intracellular concentration of labile Zn^{2+} in situ using growth plate cartilage tissue slices. Slices of tissue approximately 0.25 mm in thickness were collected from the upper region of the growth plate and placed in HBSS in glass coverslip chambers. The tissue slices were incubated in sterile HBSS for 3 h at 37°C in the presence or absence of 100 μM $ZnCl_2$. The tissue slices were next rinsed three times in HBSS and incubated with 15 μM zinquin for 60 min at 37°C. The tissue slices were washed three times in fresh HBSS to remove unincorporated zinquin and examined by MPLSM as described above. In these studies, Z-sections were digitally constructed by combining the images of nine sequential confocal sections collected at 5-μm intervals in the tissue sections. Controls for these studies included tissue sections examined without zinquin present. Quantitation of intracellular Zn^{2+} in these images was achieved by comparing fluorescence values determined in each experiment to maximum and minimum florescence as measured in identically prepared tissue slices and at the same instrumental settings using the formula $[Zn^{2+}] = K_d(F - F_{min})/(F_{max} - F)$ [Grynkiewicz et al., 1987]. After subtracting the background fluorescence of unloaded cells, F_{min} was determined in zinquin-loaded tissue slices in the presence of 100 μM of the TPEN in HBSS, and F_{max} in zinquin-loaded cells exposed to the Zn^{2+} ionophore Na-pyrithione in the presence of 50 μM Zn^{2+} [Sensi et al., 1997] in HBSS. For these studies, the relative fluorescent intensities of the Z-sections was determined by comparing overall white levels in grey scale images using Adobe Photoshop software ($n = 4$). Tissue slices used for these determinations were examined at identical instrumental settings.

For detection of apoptosis in cultured chondrocytes, cells grown on 4-chamber cover glass slides were fixed in PBS buffer containing 4% paraformaldehyde for 1 h at 20°C, rinsed in PBS, and permeabilized with ice-cold 0.1% Triton X-100, 0.1% sodium citrate for 5 min. The TUNEL reaction was conducted by incubating each chamber for 1 h at 37°C with 30 μl of a solution containing 250,000 U/L terminal deoxynucleotidyl transferase (TdT), 50 μM fluorescein-dUTP, 1 mM cobalt chloride, 140 mM sodium cacodylate, 0.25 g/L bovine serum albumin, and 30 mM Tris-HCl (Boehringer Manheim, Indianapolis, IN). Negative controls consisted of the same solution without TdT. For positive controls, cells were incubated at 20°C with 1 μg/ml DNase I in 50 mM Tris-HCl (pH 7.5), 1-mM magnesium chloride, and 1 mg/ml bovine serum albumin for 15 min prior to the TUNEL reaction. After washing with PBS, the slides were equilibrated with anti-fade buffer (Molecular Probes, Eugene, OR) and mounted with GelMount. The slides were examined by confocal fluorescence microscopy on a Bio-Rad 1024MP scanning laser microscope using the 488-nm excitation line of an Argon:Krypton

laser operated at 10% power with a fluorescein emission filter.

Statistical Analysis

The results of all quantitative measurements were compared by ANOVA with Tukey's post-test using Prism software (GraphPad Software, San Diego, CA). Differences between control and experimental treatments were considered significant at $P < 0.05$.

RESULTS

Chemical analysis of chicken metatarsal bones showed that the level of total Zn in the tissues varies in the different morphological regions of the growth plate (Fig. 1). The resting/proliferative region contains high levels of Zn with significantly lower levels observed in hypertrophic cartilage. Levels increase gradually through the calcifying zone and underlying bone. In contrast, Ca levels start out low in the upper regions and increase progressively through the growth plate as the cartilage becomes mineralized.

In order to examine the movements of intracellular zinc in chondrocytes, we utilized primary cultures of chicken growth plate chondrocytes, which undergo mineral deposition [Wu et al., 1989]. Upon attainment of confluency, the chondrocytes develop locally into multicellular nodules leading to matrix calcification. In this culture system the chondrocytes are observed to undergo the same progressive development and differentiation events known to occur in vivo [Wu et al., 1995]

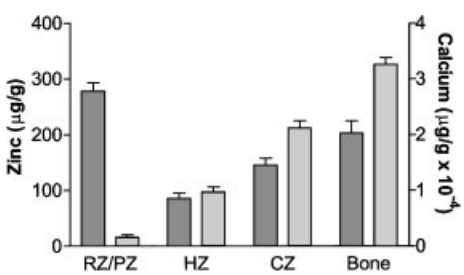

Fig. 1. Zonal analysis of zinc (dark bars) and calcium (light bars) in the epiphyseal growth plate of chicken metatarsal bone. Slices of each region were collected and were digested in nitric acid and analyzed by atomic absorption spectroscopy. Values represent the mean ± SE for five aliquots taken from a pooled tissue sample. Resting/proliferative zone cartilage (RZ/PZ); hypertrophic cartilage (HZ); calcifying cartilage (CZ); underlying bone (bone).

and will spontaneously undergo apoptosis as indicated by the TUNEL assay (Fig. 2A) beginning between 24 and 28 days of culture. The number of cells undergoing apoptosis at any one time is small but increases with age of the culture at least up to day 35 of culture. Treatment of chondrocyte cultures for 24 h with the apoptosis inducers dexamethasone (1 μM), SNP (50 μM), or Ca^{2+} ionophore A23187 (10 nM) increased the number of apoptotic cells in the cultures (Fig. 2B,D). Apoptosis was also stimulated by treatment of cultures with the membrane permeant Zn^{2+}-chelator TPEN (Fig. 2C). We did not see any changes in TUNEL staining as a result of short-term treatment with the Zn^{2+} ionophore pyrithione.

In order to determine if changes in intracellular Zn^{2+} concentrations could be measured, chondrocyte cultures were treated with Zn^{2+}, Zn^{2+} plus the Zn^{2+}-ionophore pyrithione, or the membrane permeant Zn^{2+} chelator TPEN (all at 100 μM) added to the culture media. Short-term exposure to Zn^{2+}, both in the presence and absence of pyrithione resulted in significant increases in cytosolic Zn^{2+} (Fig. 3). Treatment with TPEN rapidly reduced levels of labile Zn^{2+}. The dissociation constant (K_d) for the TSQ:Zn complex in 10 mM Tris buffer used to harvest cultured chondrocytes in these experiments was determined by Hill plot analysis to be 1.02×10^{-6}. Treatment of cultures with apoptosis inducers for 24 h resulted in significantly reduced levels of intracellular Zn^{2+} (Fig. 4). Levels of DNA and intracellular protein in these cells were slightly reduced by the apoptosis inducers (Fig. 5A,B). Differences in total DNA were significant for dexamethasone only ($P < 0.05$) while both dexamethasone and ionophore A23187 produced significant reductions in cellular protein ($P < 0.05$). Metallothionein levels (Fig. 5C) were significantly reduced by both sodium nitopruside and ionophore A23187 treatment ($P < 0.01$). Levels of DNA, cellular protein, and metallothionein were not significantly affected by short-term (1–3 h) treatment with apoptotic agents (not shown).

We used zinquin ester, a membrane-permeant derivative of TSQ, to visualize labile Zn^{2+} in live cultured chondrocytes (Fig. 6). Examination of control zinquin-loaded cells revealed a low background fluorescence with some cells showing localized regions with higher Zn^{2+} levels approaching the mid-range of relative fluorescence intensity (Fig. 6A). Cells

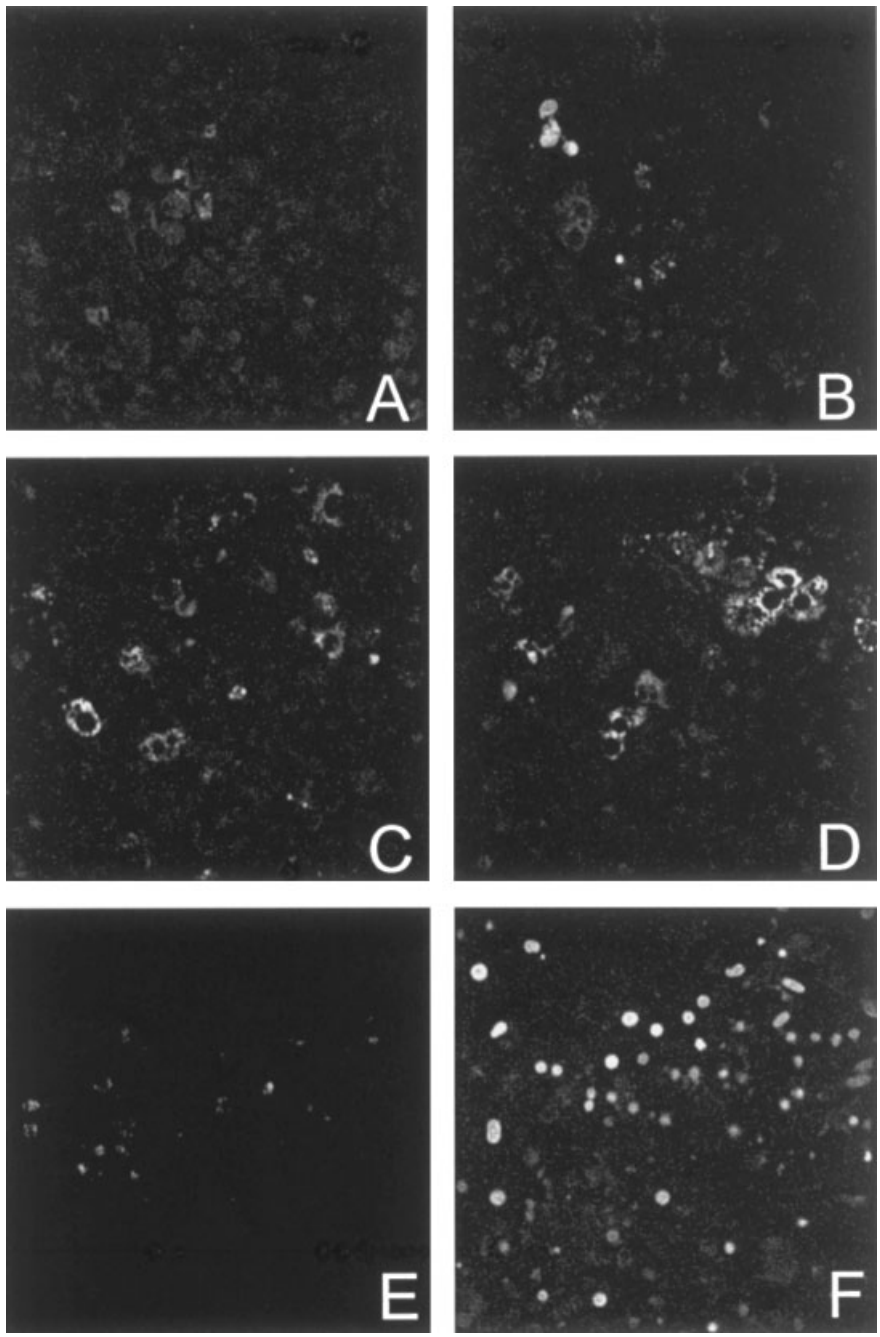

Fig. 2. Apoptotic cultured chondrocytes as detected by TUNEL reaction. Chondrocytes were cultured on glass chamber slides for 35 days in DMEM + 10% FBS, fixed with paraformaldehyde, and permeabilized with 0.1% triton X-100 prior to TUNEL reaction. **A**: apoptotic chondrocytes from 35 day old cultures; (**B**) after 24 h treatment with 10 nM ionophore A23187; (**C**) after 24 h treatment with 50 μM TPEN; (**D**) after 24 h treatment with 50 μM SNP; (**E**) negative control; (**F**) positive control treated with DNase prior to TUNEL reaction.

that were not loaded with zinquin showed very little or no background fluorescence (not shown). Zn^{2+} loaded cells showed uniformly higher relative zinquin fluorescence (Fig. 6B). Treatment of Zn^{2+}-loaded cells with TPEN quenched zinquin fluorescence (Fig. 6C). This experiment confirmed that the zinquin fluorescence we observed in chondrocytes is due to the presence of intracellular Zn^{2+}. We also used zinquin to examine cells treated overnight with a range of Zn (0–400 μM) added to the culture media. These cells, when treated with Zinquin showed

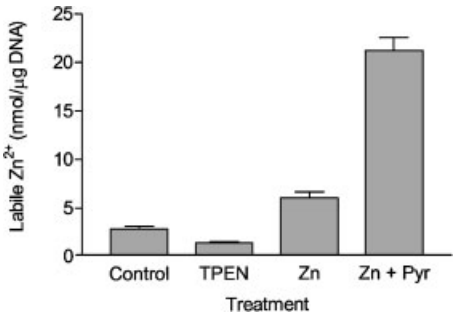

Fig. 3. Effect of 1 h incubation with 100 μM TPEN, 100 μM Zn^{2+}, or 100 μM Zn^{2+} plus 100 μM pyrithione on cytosolic labile Zn^{2+} levels in 26 day old cultured chondrocytes as determined by TSQ fluorescence assay. Values are mean ± SE from four culture dishes.

a dose-dependent increase in relative fluorescence (Fig. 7).

We have also examined changes in intracellular Zn^{2+} with zinquin after short-term (3 h) and long-term (24 h) exposure of chondrocytes to several known apoptosis inducing agents. In short-term treatments, dexamethasone treatment (Fig. 8C) resulted in little overall change in intracellular Zn^{2+} relative to controls (Fig. 8A) but showed localized increases in fluorescence in some cells. Treatment of chondrocytes with the nitric oxide generating agent SNP resulted in increased overall zinquin fluorescence (Fig. 8B) as well as numerous bright "hot spots." Some cells appeared to be disintegrating into numerous brightly fluorescent apoptotic bodies. The calcium ionophore A23187 (Fig. 8D) also resulted in a general increase in zinquin fluorescence suggesting a displacement of Zn^{2+} from intracellular binding sites. TPEN

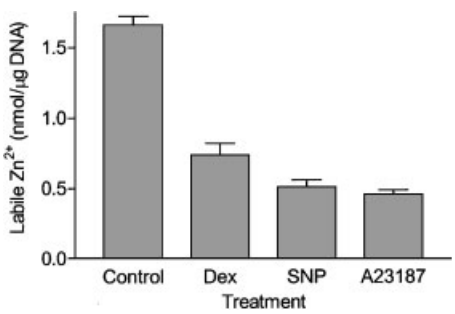

Fig. 4. Effect of 24 h incubation with dexamethasone (1 μM), sodium nitroprusside (50 μM), and ionophore A23187 (10 nM) on cytosolic labile Zn^{2+} levels in 24 day old cultured chondrocytes as determined by TSQ fluorescence assay. Values are mean ± SE from four culture dishes.

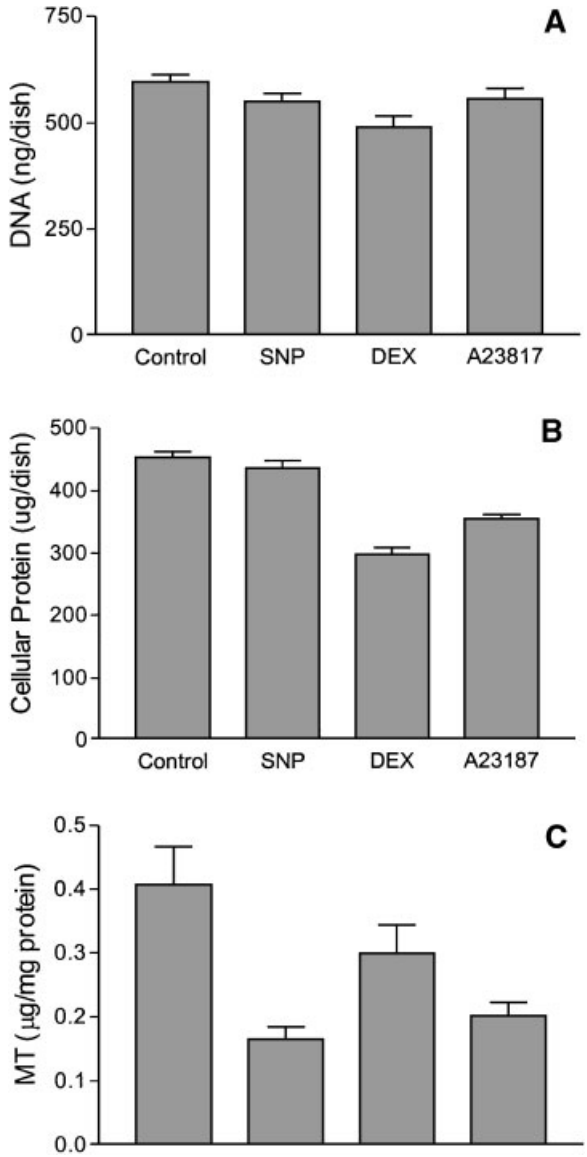

Fig. 5. Effect of 24 h treatment with apoptosis inducers (see Fig. 4) on DNA (**A**), cytosolic protein (**B**), and metallothionein (**C**) levels in 24 day old cultured chondrocytes. Values are mean ± SE from four culture dishes.

quenched the fluorescence caused by A23187 treatment indicating that it was due to Zn^{2+} and not Ca^{2+} (not shown). Long-term (24 h) treatment of chondrocytes with apoptosis inducers resulted in reduced overall zinquin fluorescence (not shown).

By Hill plot analysis the Zn^{2+}:zinquin dissociation (K_d) constant under intracellular conditions was determined to be 1.51×10^{-6} (Fig. 9). Using this value we have made an initial estimate of chondrocyte cytosolic labile

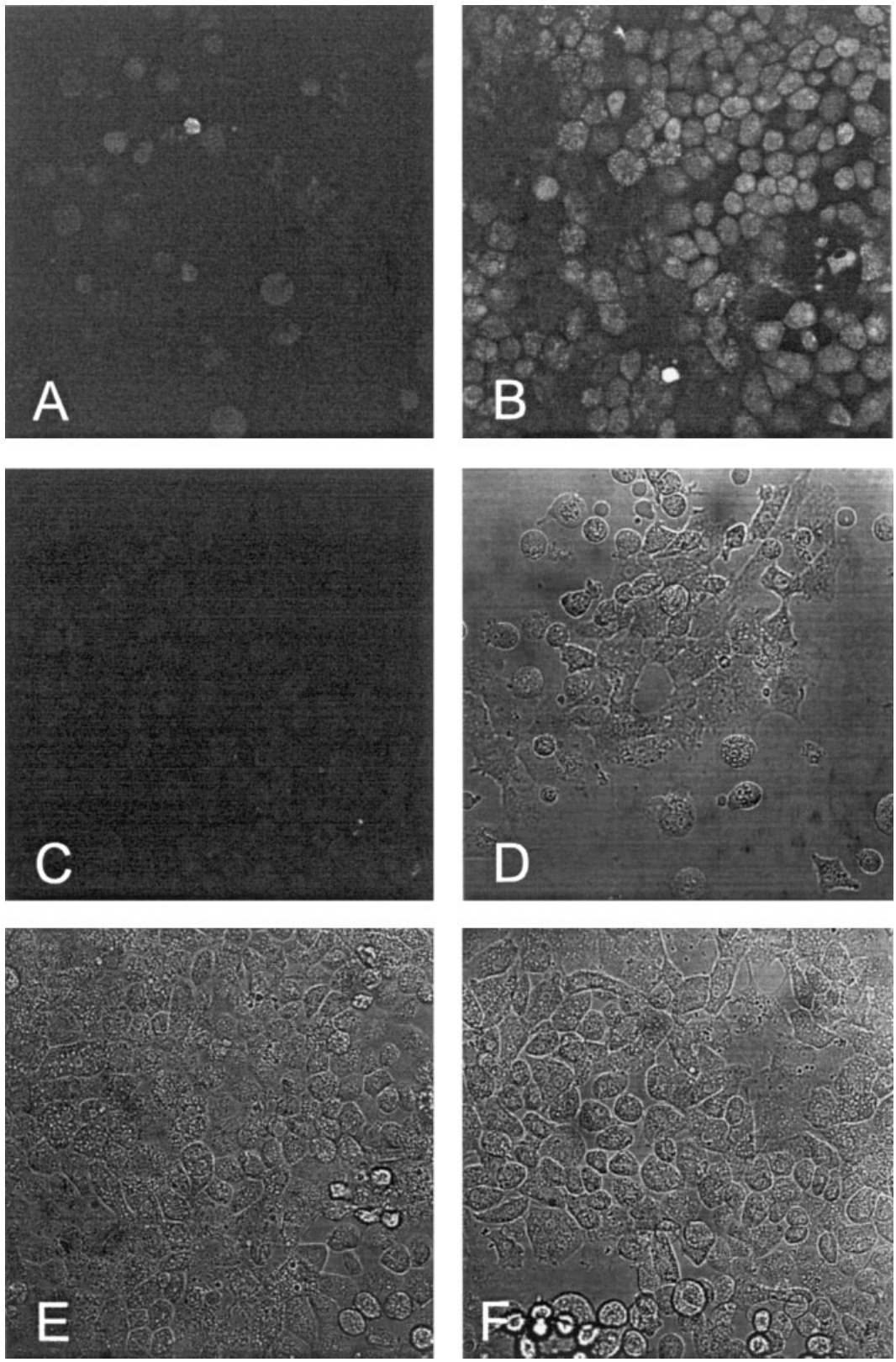

Fig. 6. MPLSM imaging of intracellular labile Zn^{2+} in 24 day old cultured chondrocytes using zinquin. Cell cultures were treated with 15 μM zinquin for 60 min at 37°C and washed with buffer prior to imaging on a Bio-Rad MPLSM system using multiphoton Ti:Sapphire laser excitation at 770 nm. Control cells (**A**) show low but variable fluorescence with localized regions of higher Zn^{2+}. Cells loaded with Zn^{2+} (**B**) by treatment with Zn^{2+} ionophore pyrithione (10 μM) and Zn^{2+} (100 μM) for 1 h show much higher overall fluorescence. Treatment of Zn^{2+}-loaded cells with TPEN (**C**) quenched zinquin fluorescence to almost background levels. **Panels D–F** are the corresponding bright field images for A–C respectively. Magnification, 200×.

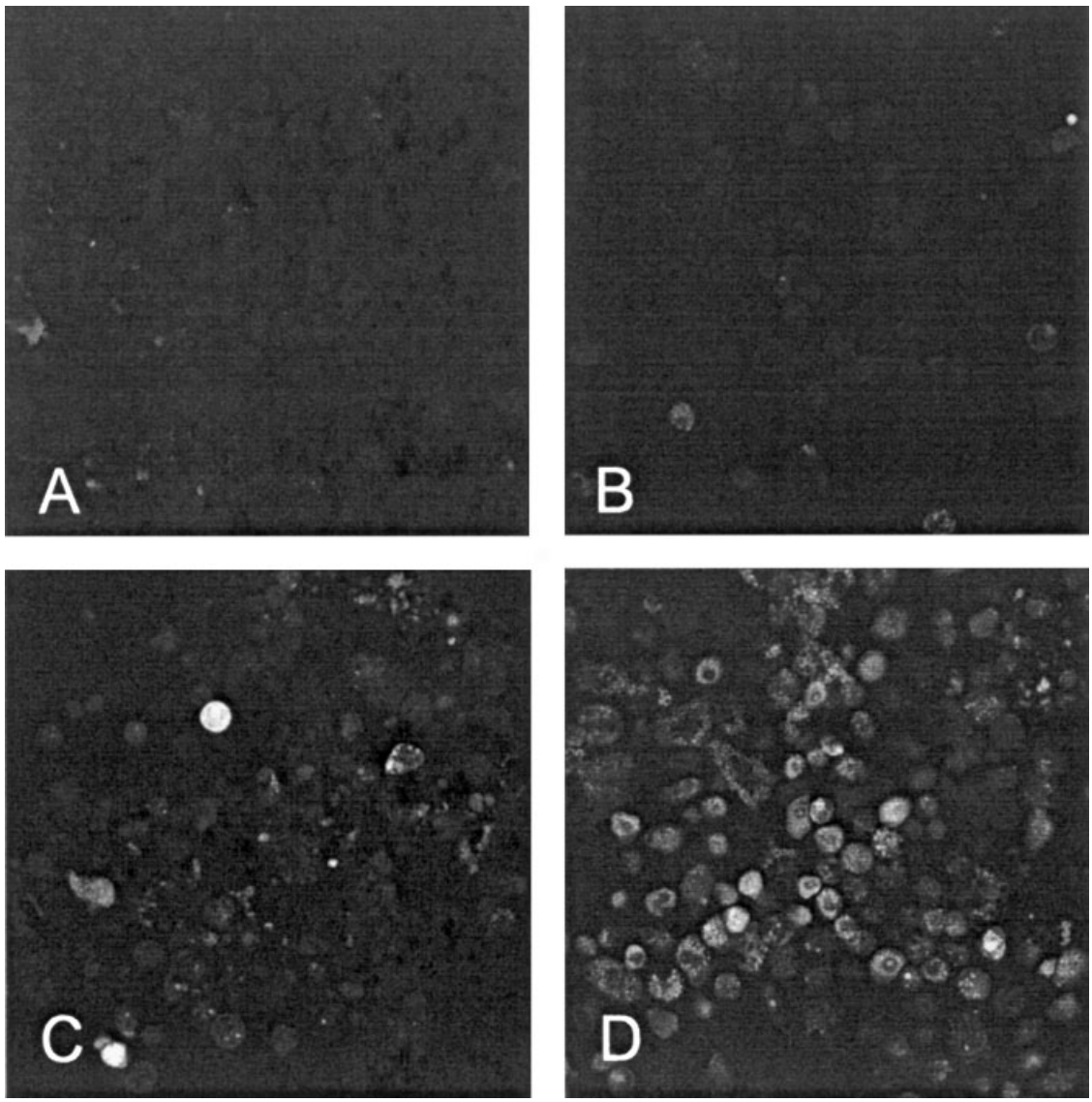

Fig. 7. MPLSM imaging of intracellular labile Zn^{2+} in 25 day old cultured chondrocytes treated overnight with graded concentrations of Zn^{2+} prior to zinquin loading. Control (**A**); 20 μM Zn^{2+} (**B**); 100 μM Zn^{2+} (**C**); 400 μM Zn^{2+} (**D**). Magnification, 200×.

Zn^{2+} of 0.27 ± 0.09 μM in growth plate cartilage tissue slices (Fig. 10) based on the overall fluorescence intensity of MPLSM images. After incubation of the tissue slices with 100 μM Zn for 3 h, labile Zn^{2+} rose approximately eightfold to 2.11 ± 0.49 μM.

DISCUSSION

The epiphyseal growth plate of chicken bones was found to contain relatively high levels of zinc with significant regional differences in the distribution of Zn^{2+} throughout the tissue. High levels of Zn^{2+} found in the proliferative zone are consistent with the known requirement for this metal ion in cell division [MacDonald, 2000]. Studies with cultured chondrocytes have shown that Zn^{2+} stimulates cell proliferation [Litchfield et al., 1998; Rodriguez and Rosselet, 2001]. Similarly, the growth plates of animals fed Zn-deficient diets show impaired development and reduced cell proliferation [Rossi et al., 2000; Wang et al., 2002]. The approximate threefold reduction in zinc observed in the hypertrophic region of the growth plate relative to the proliferative zone suggests that zinc

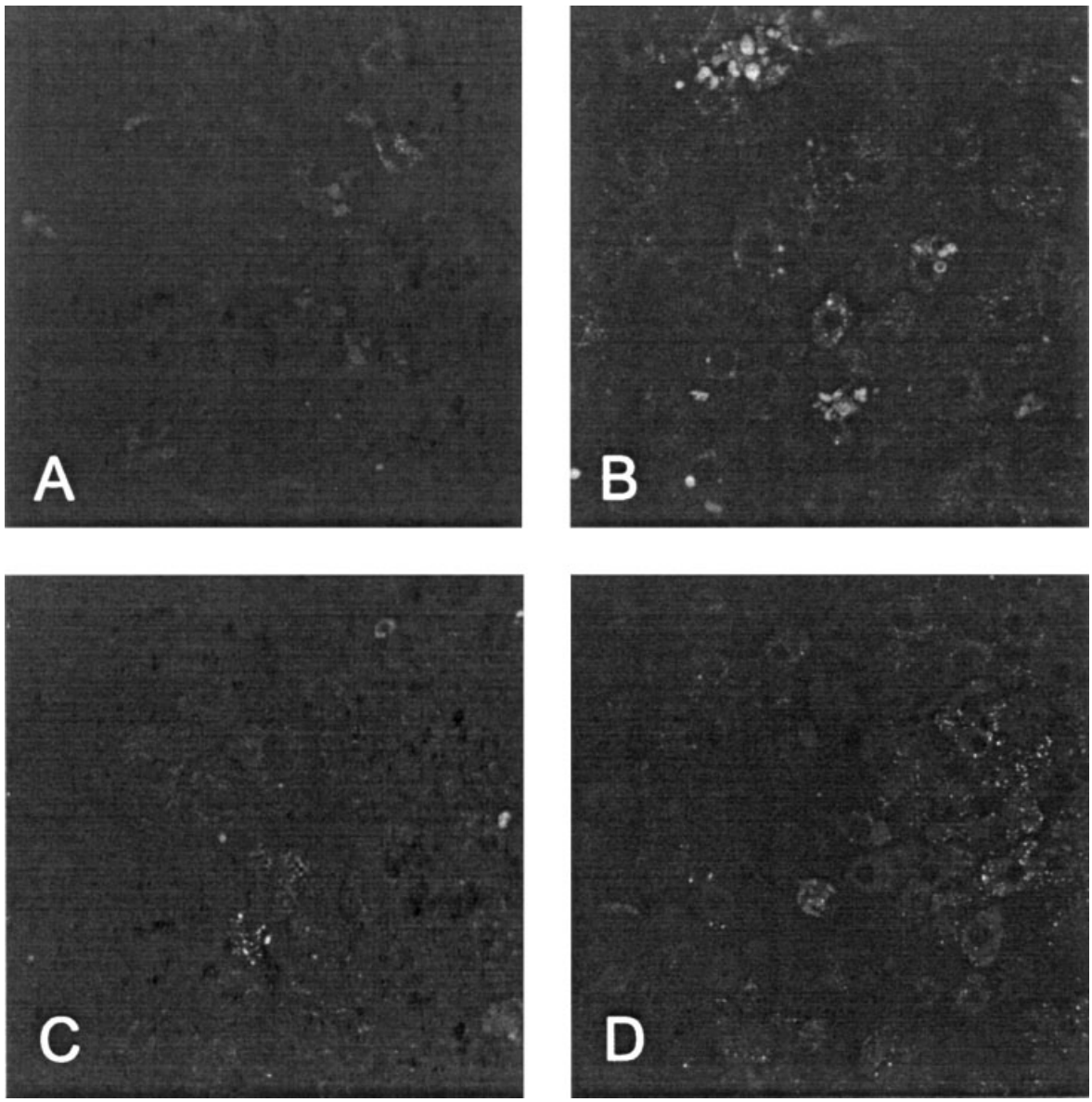

Fig. 8. MPLSM imaging of intracellular labile Zn^{2+} in 26 day old chondrocyte cultures after 3 h exposure to apoptosis inducing agents. Control (**A**); sodium nitroprusside, 50 μM (**B**); dexamethasone, 100 nM (**C**); and ionophore A23187, 10 nM (**D**). Following treatment, cells were incubated with 15 μM zinquin ester for 60 min at 37°C and washed with buffer prior to imaging on a Bio-Rad MPLSM system using multiphoton Ti:Saphire laser excitation at 770 nm. Magnification, 400×.

is lost from cells during this stage of development. It is not clear if the increasing levels of Zn^{2+} appearing in the calcifying zone and bone are derived from Zn^{2+} lost from the proliferative zone or circulating fluids. We have previously isolated functional MV from chicken epiphyseal growth plate and have subjected them to elemental analysis [Sauer et al., 1989]. MV were found to contain relatively high concentrations of Zn (1.58 μmol/g MV). MV shed by the hypertrophic chondrocytes could thus provide a vehicle for efflux of cellular Zn^{2+}. At least 40% of the Zn in MV was labile and readily extractable in isosmotic buffers. Analysis of the nucleational core complex of MV showed that most of the non-labile Zn^{2+} was associated with non-crystalline calcium phosphate, phosphatidylserine and annexin [Wu et al.,

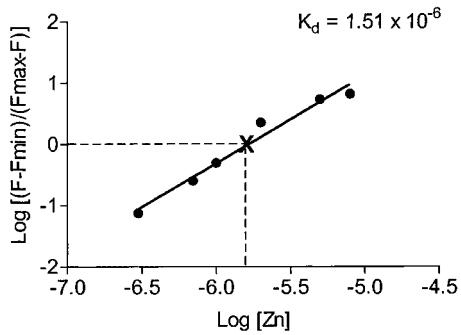

Fig. 9. Hill plot determination of K_d for the fluorescent probe zinquin and Zn^{2+} in physiological buffer designed to mimic intracellular conditions (120 mM KCl; 30 mM NaCl; 1 mM $MgSO_4$; 10 mM MOPS, pH 7.2; 1 μg/ml BSA). This plot gives an intercept of -5.82 ($K_d = 1.51 \times 10^{-6}$). Relative fluorescent intensities were determined with a Shimadzu RF-5301PC spectrofluorophotometer for each Zn^{2+} concentration, $[Zn^{2+}] = 0$, and at saturating $[Zn^{2+}]$ using excitation at 365 nm. Each point is the mean of three separate determinations.

1993]. These studies raised the question of how MV become pre-loaded with high levels of Zn^{2+} at the time of their release from the plasma membrane.

Just prior to the initiation of matrix calcification, chondrocytes have been shown to undergo nuclear condensation [Farnum and Wilsman, 1987], DNA fragmentation [Gibson et al., 1995; Hatori et al., 1995], changes in cellular redox state [Shapiro et al., 1982], loss of mitochondrial calcium [Brighton and Hunt, 1976], a sharp rise in cytoplasmic calcium levels [Wuthier, 1993], retraction from the pericellular matrix [Farnum and Wilsman, 1987], and blebbing of the plasma membrane giving rise to the formation of MV [Anderson, 1969]. These events are all indicative of late stages of an apoptotic cascade [Kroemer et al., 1995]. Morphological evidence also indicates that terminally differentiated hypertrophic chondrocytes undergo apoptosis [Zenmyo et al., 1996; Roach, 1997]. MV contain high levels of non-crystalline Ca^{2+} and P_i [Wuthier, 1977] and are enriched in cholesterol and acidic phospholipids, particularly phosphatidylserine [Wuthier, 1975], of which approximately 25% is localized on the outer surface [Majeska et al., 1979]. Together, these studies support an apoptotic origin for MV.

Changes in the intracellular distribution of Zn^{2+} have been shown to accompany apoptosis in a variety of cells [Truong-Tran et al., 2000]. In the present study, Zn^{2+} fluxes were found to occur in cultured chondrocytes treated with several well-known chemical inducers of apoptosis. Initially, we confirmed that we could detect changes in intracellular labile Zn^{2+} using fluorescent measurements after manipulating Zn^{2+} levels through the use of a chelator (TPEN) or Zn^{2+} ionophore (pyrithione). Additionally, exposing chondrocytes to elevated exogenous concentrations of Zn^{2+} for 24 h resulted in dose-dependent increases in cellular labile Zn^{2+} (Fig. 7). Short-term (3 h) exposure of cells to the apoptotic agents SNP, ionophore A23187, or dexamethasone resulted in detectable increases in cellular labile Zn^{2+}. These increases were transient, however, as longer (24 h) exposure to these chemicals resulted in significant declines in cellular labile Zn^{2+} (Fig. 4). It should be noted that our fluorescent measurements detect labile (free ionic or loosely bound) Zn^{2+} and not total cellular zinc. Most metal binding proteins have greater affinity for Zn^{2+} ($K_d = 10^{-8}$ to 10^{-10}) than that of TSQ or zinquin. Hence, tightly bound Zn^{2+} would not be detected in our measurements. The zinquin experiments with apoptosis inducers were carried out in metal free buffer. Hence, Zn^{2+} appearing in the labile cytosolic labile pool in the MPLSM images must be derived from high affinity intracellular ligands such as metalloproteins. These findings strongly indicate that Zn^{2+} fluxes occurring during chondrocyte apoptosis result from mobilization of Zn^{2+} from intracellular sites. The identity of these intracellular Zn^{2+} binding sites is not known but likely includes the cytosolic metal-binding protein metallothionein.

Metallothioneins (MT) are a family of low molecular weight (≙6800 Da), cysteine-rich metal-binding proteins which are inducible by exposure to metals, such as zinc or cadmium, as well as a number of hormones and cytokines [Bremner and Beattie, 1990]. The metal-binding capacity of MT is highly variable but most vertebrate forms can bind up to 7 metal atoms per molecule. Each metal ion bound is complexed with the sulfhydryl group of two or more of the numerous cysteine residues. In normal cells, MT appears to have a number of functions which include serving as an intracellular storage site for zinc and copper, protection of the cells from oxidative injury by scavenging free-radicals, and acting in protein synthesis by directly transferring bound metals to apo-enzymes. In addition, when it is induced by exposure to metals, MT provides tolerance to metal toxicity by sequestering the toxic metal

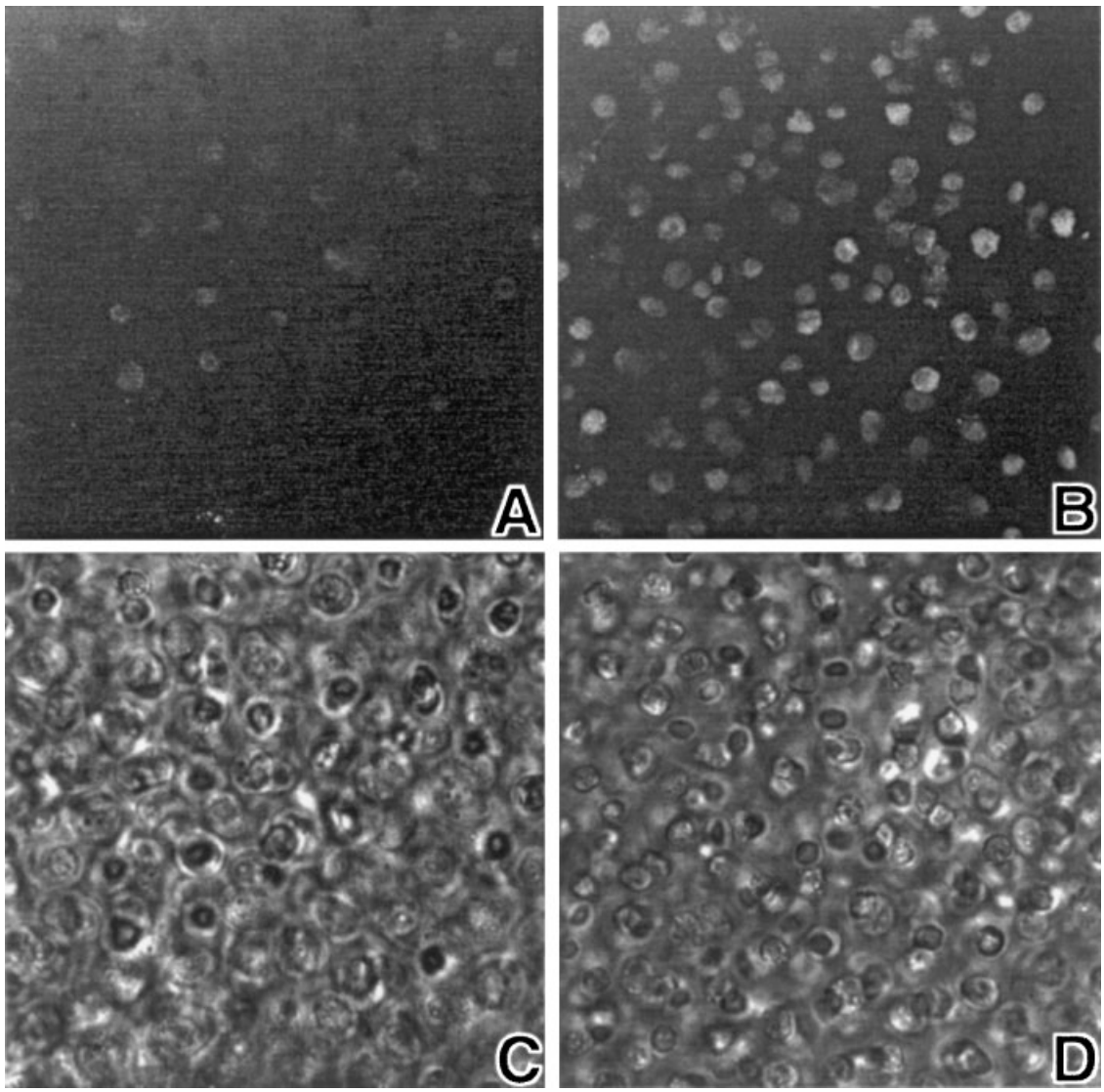

Fig. 10. Multi-photon laser scanning microscopic images of intracellular labile Zn^{2+} in slices of growth plate cartilage tissue as indicated by zinquin fluorescence. Control tissue slices (**A**) show lower levels of intracellular Zn^{2+} (0.27 ± 0.09 μM) than slices exposed to 100 μM Zn for 3 h (**B**) (2.11 ± 0.49 μM). Images shown are Z-sections composed of nine separate 5 μm slices. Corresponding bright field images of the tissue sections are shown in **panels C** and **D**. Quantitation of Zn^{2+} is by the formula: $[Zn^{2+}] = K_d(F - F_{min})/F_{max} - F)$ using overall white levels determined for whole images. Magnification, 100×.

ions, lowering the metal concentrations at intracellular target sites. It has been suggested that because of the abundance of oxidizable thiol groups, the primary role of metallothioneins in normal cell physiology may be to act as a defense mechanism against the damaging effects of oxygen, nitrogen, and carbon-centered free radicals generated by normal metabolic processes [Lazo and Pitt, 1995]. During the apoptotic cascade, reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radicals, and nitric oxide are produced which react with intracellular membranes leading to the final degradation of the cells [Halliwell and Gutteridge, 1990; Hockenbery, 1995; Kroemer et al., 1995]. MT may thus function to prevent the onset cell death by buffering the levels of ROS in the cell. In the present study, the greatest fluxes of Zn^{2+} were observed with the NO generating chemical SNP. Of the chemical inducers tested, SNP also caused the greatest decline in MT levels after 24 h of treatment. The Zn-S thiolate

bonds of metallothionein are highly sensitive to oxidation by ROS, which results in the release of bound Zn^{2+} [Fliss and Menard, 1992; Kroncke, 1994; Misra et al., 1996]. In chondrocytes, the oxidation of MT by ROS during apoptosis may be the cause of the elevated levels of Zn^{2+} we have observed in MV and thus provide a mechanism for the transfer of metal ions to the extracellular matrix. During apoptosis, Zn^{2+} may be released from other intracellular macromolecules as well. For example, it has recently been shown that DNA-damaging agents produce transient Zn^{2+} fluxes that were correlated with the onset of caspace activation in lymphoma cells [Smith et al., 2002].

We previously observed age-specific differences in the basal and inducible levels of MT in chondrocyte cell cultures [Sauer et al., 1998]. During the early stages of culture, corresponding developmentally to proliferative chondrocytes basal levels of MT were higher than at later stages of the culture during active matrix mineralization. These findings suggest that changes in cytosolic levels of MT and Zn^{2+} may have important consequences to the cell differentiation pathway and the onset of chondrocyte apoptosis. Specifically, increased production of NO or other oxidants as chondrocytes hypertrophy [Nishida et al., 2001], may deplete the cells of MT and other anti-oxidants leading to the onset of apoptosis. Dexamethasone is known to induce MT expression in many cell types. In a previous study [Sauer et al., 1998], treatment of chondrocyte cultures with dexamethasone, resulted in lower levels of MT despite an increase in the expression of MT mRNA. This suggests that the apoptotic effects of dexamethasone result in a net loss of MT from the cell despite additional MT expression induced by the steroid. Zn^{2+} ions released by MT degradation would then appear in the labile Zn^{2+} pool.

On average, approximately 30% of total cellular zinc is located within the nucleus, 50–60% is in the cytosol, with the remainder associated with the plasma membrane and intracellular organelles [Smeyers-Verbeke et al., 1977]. Within each of these compartments, the Zn^{2+} ions are principally bound to various metalloenzymes, Zn^{2+}-binding proteins, and nucleotides [Vallee and Falchuk, 1993]. Just what regulates the transfer of Zn^{2+} ions between the intracellular compartments and from one macromolecule to another is not known, but this information is critical to our understanding of the many Zn^{2+}-dependent cell processes. The cytosol is the central compartment in intracellular metal metabolism because it is: 1) the largest compartment; 2) the location where newly accumulated Zn^{2+} first appears; and 3) the primary storage site for Zn^{2+} ions [Cousins, 1985]. This compartment includes a labile pool consisting of free and loosely complexed Zn^{2+} ions, a soluble metalloprotein pool (primarily cytosolic enzymes such as carbonic anhydrase), and a metallothionein pool, which represents the primary storage form of zinc in the cell. Any transfer of Zn^{2+} ions between subcellular compartments or macromolecules requires the participation of the cytosolic Zn^{2+} pools. While the levels of free Zn^{2+} in living cells have been estimated to be in the nanamolar range [Adebodun and Post, 1995; Atar et al., 1995; Brand and Kleineke, 1996; Sensi et al., 1997], the concentration of cytosolic Zn^{2+} in the labile pool that includes weekly bound metal ions is more likely in the range of 1–10 μM [Pattison and Cousins, 1986; Brand and Kleineke, 1996]. The fluorophores TSQ and zinquin are ideally suited for measurements of labile Zn^{2+} in this concentration range. For cytosolic labile Zn^{2+} measurements in living cells, zinquin must be used for short-term (1–3 h) measurements as with time it becomes sequestered into cytoplasmic vesicles [Palmiter et al., 1996].

Two-photon or MPLSM achieves excitation of a fluorophore by the simultaneous (within 1 fs) absorption of two (or more) low energy infrared photons [Potter, 1996]. The Ti:Sapphire laser is tunable across a broad range of wavelengths so can be used with all common fluorophores. Because the two-photon effect occurs only at the point of focus, photobleaching and phototoxicity, which are especially troublesome with UV excitable dyes, are greatly reduced relative to typical confocal microscopy. There is no out of focus fluorescence in MPLSM resulting in greatly enhanced signal-to-noise ratio and more accurate quantitation of intracellular probes. The longer wavelengths employed in MPLSM allow a greater depth of imaging than is achievable in conventional confocal laser microscopy and give a better capability of imaging deep within slices of cartilage tissue. Using the capabilities of MPLSM and zinquin we were able to estimate the concentration of Zn^{2+} in the cytosolic labile pool of chondrocytes at approximately 0.3 μM. This value is slightly lower than

previous determinations of Zn^{2+} using zinquin in hepatocytes [Brand and Kleineke, 1996] and significantly lower than what has been reported in thymocytes [Zalewski et al., 1993]. Additionally, based upon comparisons of the zinquin images for chondrocytes exposed to apoptosis inducers with cells loaded by pyrithione/Zn^{2+} treatment we can estimate that the magnitude of the Zn^{2+} fluxes associated with apoptosis is in the micromolar range. It should be noted that these estimates are based on overall fluorescent intensities of whole images. More detailed image analysis studies will be required to relate actual intracellular labile Zn^{2+} concentrations with physiological changes in the cell.

It is widely recognized that zinc has an important role in regulating apoptosis [Truong-Tran et al., 2000]. Suppression of apoptosis by Zn^{2+} has been attributed to the specific inhibition of a Ca^{2+}-dependent endonuclease which is responsible for DNA fragmentation [Gaido and Cidlowski, 1991]. Subsequently, it was shown that the removal of intracellular free Zn^{2+} by chelation induced apoptosis in thymocytes [McCabe et al., 1993]. There are other steps of the apoptosis process that are more susceptible to modulation by Zn^{2+} than endonuclease activity [Wolf et al., 1997]. For example, caspase-3, which acts upstream of endonuclease, can be inhibited by micromolar concentrations of Zn^{2+} [Perry et al., 1997] and in some cells apoptosis induced by Zn^{2+}-chelation results from the activation of caspace-3 [Chimienti et al., 2001]. The present study suggests that in chondrocytes, the release of Zn^{2+} from these or other intracellular regulatory sites results in a flux of Zn^{2+} ions from the cells to the extracellular matrix. Zn^{2+} fluxes occurred early in the apoptosis process before any detectable changes in DNA, cellular protein, or MT levels occurred. The identification of the Zn^{2+}-dependent sites which regulate apoptosis in chondrocytes awaits further study. While apoptosis is a normal part of bone growth and development, studies have shown that dysregulation of this process can lead to a variety of bone and joint pathologies including chondrodsyplasia, osteoporosis, osteoarthritis, and rheumatoid arthritis [Hashimoto et al., 1998a,b; Horton et al., 1998; Hock, 1999; Kim and Song, 1999]. Prevention of apoptosis through manipulation of intracellular Zn^{2+} levels may thus provide an effective means for treating these debilitating skeletal diseases.

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