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Induction and Characterization of Metallothionein in Chicken Epiphyseal Growth Plate Cartilage Chondrocytes

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Abstract Following exposure to cadmium or zinc, chickens were sacrificed and the liver, kidney, and bone epiphyseal growth plates harvested. When cytosolic extracts of the growth plate cartilage were fractionated by gel filtration chromatography, a protein with high metal-binding capacity and low ultraviolet (UV) absorbance eluted in the same position as liver metallothionein (MT) and a MT standard. Cd or Zn treatment resulted in a 25-fold or 5-fold induction in growth plate MT, respectively. In liver the greatest level of MT induction was seen with short-term Cd exposures. In contrast, MT levels in the growth plate increased as the duration of Cd exposure increased. Induction of MT in growth plate chondrocyte cell cultures was observed for media Cd concentrations of ≥0.1 μM and Zn concentrations of ≥100 μM. Basal and inducible levels of MT declined through the culture period and were lowest in the terminally differentiated mineralized late stages of the culture. Alkaline phosphatase activity was also lowest in the late-stage cultures, while total cellular protein increased throughout the culture period. Treatment of chondrocytes with Zn prior to Cd exposure resulted in a protective induction of MT. Pre-treatment of chondrocytes with dexamethasone resulted in suppressed synthesis of MT upon Cd exposure and greater Cd toxicity. Both Cd and Zn resulted in significantly increased levels of MT mRNA in chondrocyte cell cultures. Dexamethasone treatment resulted in an approximate 2- to 3-fold increase in MT mRNA. This is contrary to the finding that MT protein levels were decreased by dexamethasone. The findings suggest that an increased rate of MT degradation in dexamethasone-treated and late-stage chondrocyte cultures may be associated with the terminally differentiated phenotype. J. Cell. Biochem. 68:110–120, 1998.

Key words: cadmium; zinc; cell culture; mineralization

Endochondral bone formation represents the primary mechanism of vertebrate skeletal development and is the means by which longitudinal bone growth occurs in the epiphyseal growth plate [Hunziker, 1994]. During this process, growth plate chondrocytes progress through a number of distinct maturational stages eventually becoming enlarged hypertrophic cells. Progression to the hypertrophic phenotype is accompanied by the release of extracellular matrix vesicles from the chondrocyte cell membrane [Anderson, 1995]. The matrix vesicles are the initial sites of calcium phosphate mineral formation and thereby trigger calcification of the extracellular matrix [Wuthier, 1988]. Previously we have found that matrix vesicles contain high levels of labile Zn^{2+} that appears to function in the regulation of vesicle mineralization [Sauer et al., 1989]. The mechanism by which matrix vesicles become enriched with Zn^{2+} ions prior to their release is not currently known. A large number of studies have indicated that skeletal tissues such as bone and cartilage are often targets of metal ion toxicity [Wallach and Chaumser, 1990]. Cadmium exposure, for example, has been shown to cause congenital bone abnormalities [Gale and Ferm, 1973], decreased bone mineral content [Bhattacharyya et al., 1988], reduced thickness of epiphyseal growth plate cartilage [Imai et al., 1995], and increased incidence of bone fractures [Nogawa, 1981]. Despite the many known effects of trace metal deficiency and toxicity on skeletal tis-

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sues, very little is known concerning the intracellular metabolism of metal ions in mineralizing cells.

Metallothioneins (MT) are a family of low-molecular-weight (≅6,800 Da), cysteine-rich metal-binding proteins that are inducible by exposure to metals, such as zinc or cadmium, as well as a number of organic compounds, including some hormones and cytokines [Bremner and Beattie, 1990]. In normal cells, MT appears to have a number of functions, which include (1) serving as an intracellular storage site for zinc and copper; (2) protection of the cells from oxidative injury by scavenging free radicals; and (3) acting in protein synthesis by directly transferring bound metals to apo-enzymes. In addition, when it is induced by metal exposure, MT provides tolerance to metal toxicity by sequestering toxic metals, thereby lowering their concentrations at intracellular target sites. Metallothionein induction has previously been demonstrated in an osteoblastic cell line [Miyahara et al., 1986]. On the other hand, a cloned rat osteosarcoma cell line apparently lacks the ability to produce MT in response to Cd treatment [Angle et al., 1990]. Metallothionein induction has not previously been examined in growth plate cartilage.

In the present study, the induction of metallothionein was examined in avian growth plate cartilage and in primary cultures of growth plate chondrocytes. This cell culture system has been shown previously to mirror the developmental stages that occur in vivo [Wu et al., 1995]. MT levels were determined following exposure to Cd, Zn, and a variety of non-metal-inducing agents at different maturational stages of the cell cultures.

MATERIALS AND METHODS

Animals

In this study, 6- to 8-week-old broiler strain chickens (400–600 g) were obtained from a commercial chicken farm (Newberry, SC). The birds were housed together in a large chicken pen and fed Ralston Purina chicken feed and water ad libitum. Individuals were identified by numbered leg bands. Throughout the duration of the experiments lighting was maintained on a 12-h light/dark cycle and temperature kept at 20°C. The birds were acclimated to these conditions for 1 week prior to the initiation of experimental treatments.

Chemicals

Cadmium chloride, zinc chloride, dexamethasone, and retinoic acid were obtained from Sigma Chemical Co. (St. Louis, MO). $^{109}CdCl_2$ (3.27 mCi/mg) in 0.5 M HCl was obtained from E.I. DuPont de Nemours & Co. (Wilmington, DE). Sephadex G-75 resin and ultrapure Tris (Trizma) buffer were from Sigma. Purified rabbit metallothionein was a gift from F.A. Liberatore (E.I. DuPont de Nemours, N. Billerica, MA). HL-1 serum free cell culture media was purchased from Hycor Biomedical (Irvine, CA). Fetal bovine serum (FBS) was from Atlanta Biological (Atlanta, GA). All other cell culture media and reagents were from Sigma. Reagents for RNA isolation and analysis were purchased from Ambion (Austin, TX). All other chemicals were of reagent grade and were purchased from Fisher Scientific (Atlanta, GA).

Metallothionein Induction

Chickens were given subcutaneous injections of $CdCl_2$ (2 mg/kg body weight) or $ZnCl_2$ (10 mg/kg body weight) dissolved in sterile 0.9% saline. In short term experiments, injections were given daily for 3 days. For longer-term experiments injections were given every 2 days, for 13 days. Control birds received injections of saline only. Two days after the final injections, the birds were sacrificed by cervical dislocation and kidneys, liver, and growth plate cartilage harvested by dissection. Growth plate tissue was harvested from the femurs, tibiae, and tarsal bones and pooled. The pooled tissues from each treatment were homogenized under N_2 in an equal volume of ice-cold 20 mM Tris buffer (pH 8.6) containing 1 mM benzamidine and centrifuged at 100,000*g* for 30 min. Aliquots of the supernatant were taken for quantitation of metallothionein by the Cd-hemoglobin affinity assay [Eaton and Cherian, 1991] and protein by Coomassie blue binding. The remainder was used for MT isolation and characterization.

Metallothionein Isolation

MT was isolated from the tissue supernatant by ethanol precipitation using established procedures [Vasak, 1991]. The precipitated protein was dried under N_2, solubilized in 10 mM Tris–25 mM NaCl (pH 8.6), and applied to a Sephadex G-75 column (2.5 × 100 cm) equilibrated with 20 mM Tris–10 mM NaCl (pH 8.6), and eluted with the same buffer at a flow rate of

60 ml/h. The eluate was monitored at 280 and 254 nm and the metal (cadmium and zinc) content of collected fractions (10 ml) determined by atomic absorption spectroscopy. For some samples, the Cd-binding capacity of the fractions was determined using 0.2-ml aliquots by the Cd-hemoglobin affinity assay as described above.

Cell Culture

Chondrocytes were isolated from the epiphyseal growth plate cartilage of tibiae from 6- to 8-week old hybrid broiler-strain chickens as described previously [Wu et al., 1995]. Cells were plated at a density of 4.5×10^5 cells per 35-mm dish and cultured in 2 ml Dulbecco's modified Eagle medium (DMEM) containing 10% FBS at 37°C in a 5% CO_2 atmosphere. The culture medium was changed every 3 days. On day 6, the cells were switched to a 1:1 mixture of DMEM and serum-free HL-1 medium (Hycor Biomedical, Irvine, CA). From day 9 onward, the cells were given only HL-1. Ascorbate was provided from day 3 onward at a concentration of 50 µg/ml. All experiments were performed on 17- to 21-day-old cultures unless otherwise indicated. $CdCl_2$ and $ZnCl_2$ were added to the cultures from stock solutions prepared in sterile distilled water to achieve the desired concentrations with minimum dilution of the media (<1%). Dexamethasone and retinoic acid were prepared as stock solutions in ethanol and added to culture media in a minimal volume (<10 µl) to achieve the desired concentrations. Two days after the addition of inducing agents, the media was removed from the dishes and the cells were washed twice with ice-cold phosphate-buffered saline (PBS). The cells from each dish were harvested into 1.5 ml ice-cold 10 mM Tris (pH 7.4) and disrupted by sonication. Cell debris and matrix material were sedimented from the cell lysate by centrifugation at 28,000*g* for 20 min in a refrigerated microcentrifuge. Aliquots of the supernatant were used for quantitation of metallothionein, cytosolic protein, and intracellular potassium. Metal toxicity was measured by determining the levels of potassium in the cytosolic extract by atomic absorption spectrophotometry following dilution with 20 mM LiCl [Klaasen and Liu, 1991a].

Statistical Analysis

Each experimental treatment consisted of four dishes. The means of each treatment were compared using Student's t-test, assuming equal variance in most cases. For all data sets, the validity of using the equal variance assumption for the two-sample t-test was checked using the F-test, $P < 0.05$. In instances in which the ratio of the sample variances differed by <0.33 or >3.0, the heteroscedastic t-test (unequal variances) was used. Each experiment was repeated at least twice.

Analysis of MT mRNA Expression

After washing with ice-cold PBS, total RNA was isolated from chondrocyte cell cultures by the single-step guanidinium thiocyante acid-phenol method [Chomcynski and Saachi, 1987]. Precipitated RNA was resuspended in 2 M LiCl–5 mM EDTA and incubated on ice for 30 min to solubilize proteoglycans, recovered by centrifugation at 11,000*g*, washed with 70% ethanol, and finally resuspended in DEPC-ddH_20/0.1 mM EDTA. MT mRNA was quatitated by reverse transcriptase-polymerase chain reaction (RT-PCR) using the EZ rTth RNA PCR Kit (Perkin Elmer, Foster City, CA) according to manufacturers directions; 5 µg of total RNA was reverse transcribed for 60 min at 37°C and cDNA precipitated by the addition of 1/10 (v/v) of 3 M sodium acetate and 2.5 vol of ethanol. The precipitates were resuspended in ddH_2O and aliquots of the cDNA used for the PCR reaction. After an initial heating at 94°C for 5 min, amplification was conducted at 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s over 30 cycles, followed by 10 min of extension at 72°C. MT PCR primers designed from the known chicken MT DNA sequence [Andrews and Fernando, 1991] were 5′ CCT CAG GAC TGC ACT TGT GC (68–87) sense and 5′ TGG CAG CAG CTG CAC TTG CT (1385–1366) antisense, which yield a 182-bp gene product of the coding mRNA. As an internal control, primers for glyceraldehyde-3-phosphate dehydrogenase, 5′ GAC CAC TGT CCA TGC CAT CAC AGC C (575–599) sense and 5′ TCC AAA CTC ATT GTC ATA CCA GGA A (1007–983) antisense, which yield a 433-bp product [Pnabrieres et al., 1983], were included in the reaction tubes. A 10-µl aliquot of each amplified sample was analyzed using 2% agarose TBE gels and DNA detected by ethidium bromide staining.

RESULTS

Sehadex G-75 fractionation of the cytosolic extracts of growth plate cartilage from chickens

exposed to Cd or elevated levels of Zn revealed a protein with an apparent molecular weight of 10,000 kDa with low ultraviolet (UV) absorbance and high metal-binding capacity (Fig. 1) characteristic of metallothionein. MT from liver of the same birds as well as a rabbit MT standard eluted at the same position. Levels of MT in growth plate cartilage, kidney, and liver of control and metal-exposed birds were quantitated by the Cd-hemoglobin affinity assay (Table I). MT levels were very low in the control growth plate relative to levels observed in kidney and liver. Cd or Zn treatment resulted in 25- or 5-fold maximum induction respectively in growth plate tissue after a 14-day exposure. Low levels of induction were observed in the kidneys. In liver, short-term metal exposure resulted in the greatest induction of MT with lower levels observed after long-term treatment. In contrast, MT levels in growth plate tissue increased as the duration of Cd exposure increased.

In growth plate chondrocyte cell cultures, significant induction of MT was observed for added Cd concentrations of ≥0.1 μM and Zn concentrations of ≥100 μM (Fig. 2). At the higher end of each range of metal concentra-

TABLE I. Metallothionein Content of Liver, Kidney, and Growth Plate Tissue from Chickens Exposed to Cadmium or Zinc*

Treatment	Tissue	Induction period	
		3 day	14 day
Control	Liver	0.97 ± 0.11	1.43 ± 0.28
	Kidney	0.99 ± 0.09	0.62 ± 0.03
	Growth plate	0.06 ± 0.03	0.04 ± 0.01
Cadmium 1 mg/kg body weight	Liver	33.51 ± 2.11	14.45 ± 0.83
	Kidney	1.43 ± 0.21	1.70 ± 0.03
	Growth plate	0.54 ± 0.09	1.03 ± 0.09
Zinc 10 mg/kg body weight	Liver	—	22.05 ± 1.53
	Kidney	—	0.64 ± 0.14
	Growth plate	—	0.23 ± 0.02

*Chickens received intraperitoneal injections of saline with indicated metal dosage or saline only (control). Metallothionein (μg MT/mg protein ± SE) was determined by the Cd-hemoglobin binding assay.

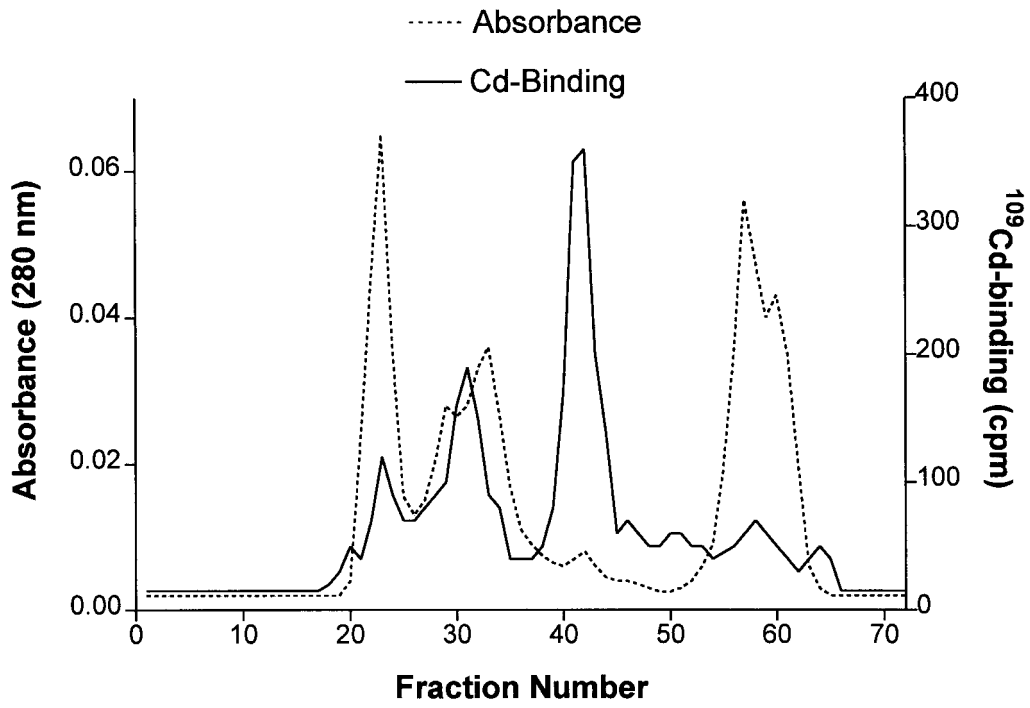

Fig. 1. Sephadex G-75 fractionation of cytosolic extract of epiphyseal growth plate tissue of chickens exposed to Cd (1 mg/kg body weight) for 17 days. The extract was eluted with 20 mM Tris–HCl, 10 mM NaCl (pH 8.6) at a flow rate of 50 ml/h. 10-ml fractions were collected from a 100-cm column. Prior to counting, aliquots of each fraction were incubated with 109_{Cd} (10,000 cpm/ml), with excess 109_{Cd} scavenged by hemoglobin addition and heat precipitation to remove the hemoglobin.

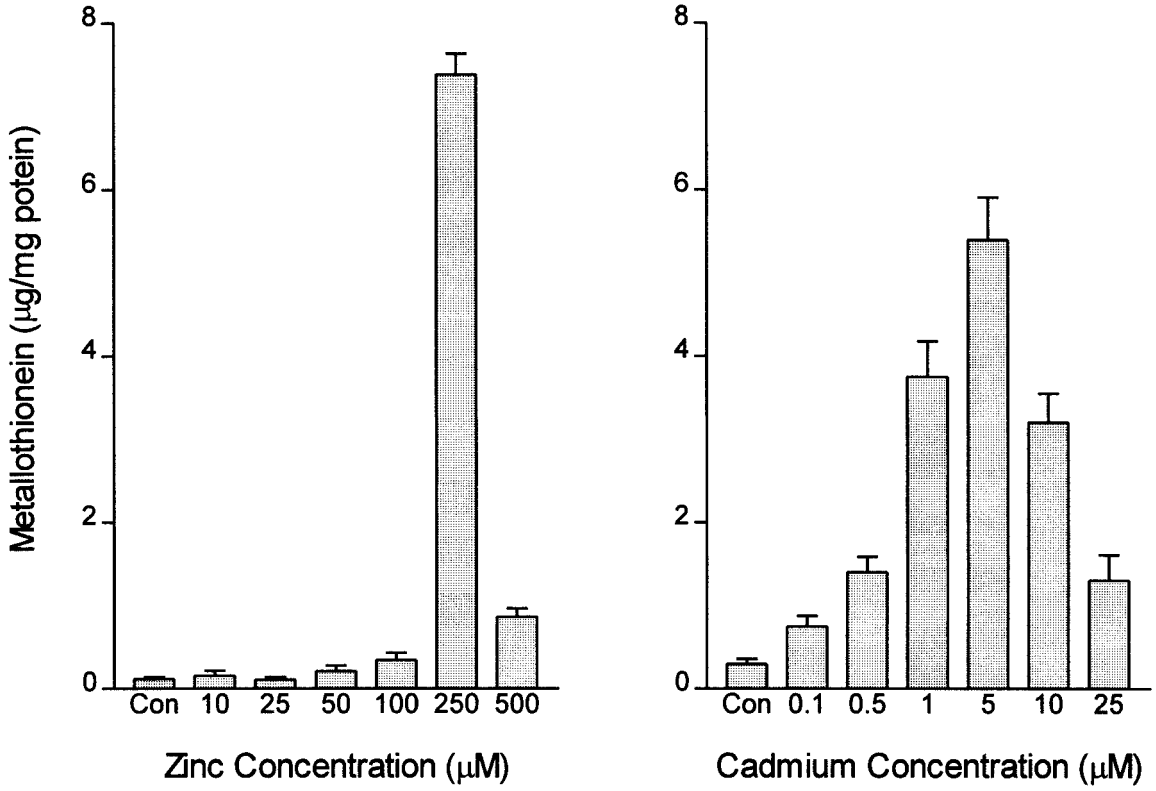

Fig. 2. Metallothionein induction in 20-day-old primary cultures of chicken growth plate chondrocytes exposed for 2 days to different concentrations of cadmium or zinc. Significant ($P < 0.01$) induction of MT was observed at cadmium concentrations of ≥0.1 μM and Zn concentrations of ≥100 μM. Metallothionein was determined by Cd-hemoglobin assay of cytosolic extracts.

tions, MT levels in the cell cultures declined as the metals became acutely toxic. Acute toxicity of metal ions was confirmed at 10 μM Cd and 400 μM Zn by measuring intracellular potassium levels (not shown). Basal levels of MT in chondrocytes receiving no treatment varied slightly between experiments but generally ranged from 0.05–0.25 μg/mg protein. The reason for this variation is unknown but is likely due to genetic, nutritional, and/or physiological differences in the chickens used as source material which were obtained at different times throughout the year. Levels of MT in the cells increased for up to 4 days following the addition of metal to the culture media (Fig. 3). In most of the studies reported here 2-day incubation periods with added metal were used. Treatment of cells with dexamethasone caused a decline in cellular MT to greater than 50% of the level observed in control cultures (Fig. 4). Continuous dexamethasone treatment caused a reduction in the levels of alkaline phosphatase and total cellular protein (Fig. 5). This observation prompted us to use only short-term (1- to 2-day) dexamethasone treatments in our experiments. Short-term treatment with dexamethasone did not cause any significant changes in alkaline phosphatase activity or cellular protein (data not shown).

Pre-treatment of chondrocytes with Zn prior to Cd resulted in the expression of very high levels of MT upon subsequent Cd exposure (Fig. 6A). The Zn-pretreated cells were more resistant to Cd toxicity than were cells that did not receive Zn pre-treatment (Fig. 6B). Pre-treatment with dexamethasone, on the other hand, suppressed the levels of MT resulting from Cd exposure and caused a slight increase in Cd toxicity. Expression of MT mRNA was examined by RT-PCR following 24-h exposure to various inducing agents (Fig. 7). Cd and Zn exposure resulted in strong expression of MT mRNA. Dexamethasone treatment also increased MT mRNA expression, though at somewhat lower levels. Retinoic acid did not cause a noticeable increase in MT mRNA. Similarly, MT protein levels were not affected by short-term exposure to retinoic acid (not shown). Cd

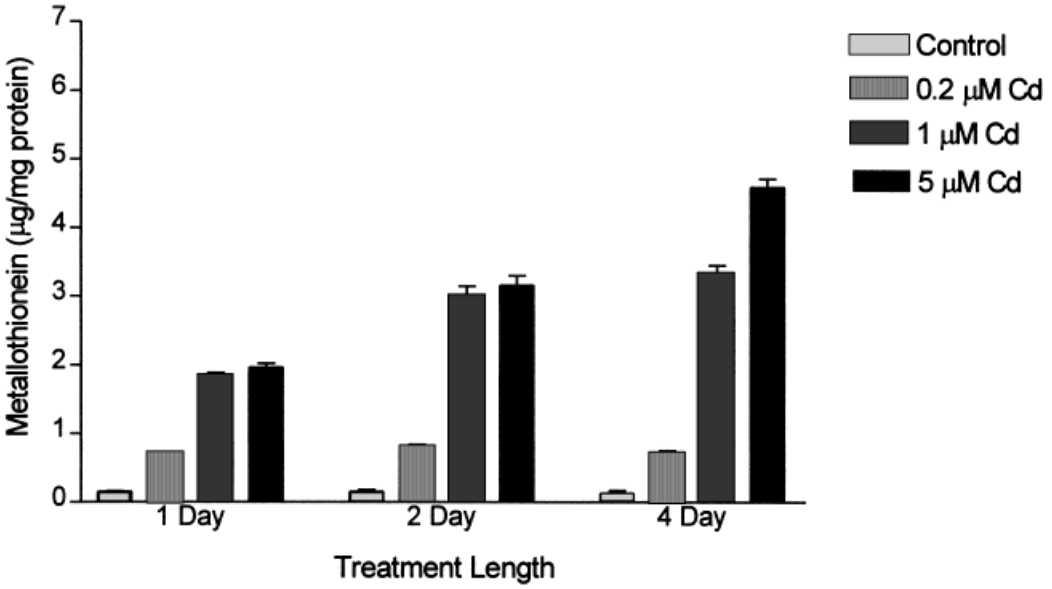

Fig. 3. Metallothionein levels in 23 day old chondrocyte cell cultures after 1–4 days of exposure to Cd. All Cd treatments resulted in significant ($P < 0.01$) induction of MT. Metallothionein was determined by Cd-hemoglobin assay of cytosolic extracts.

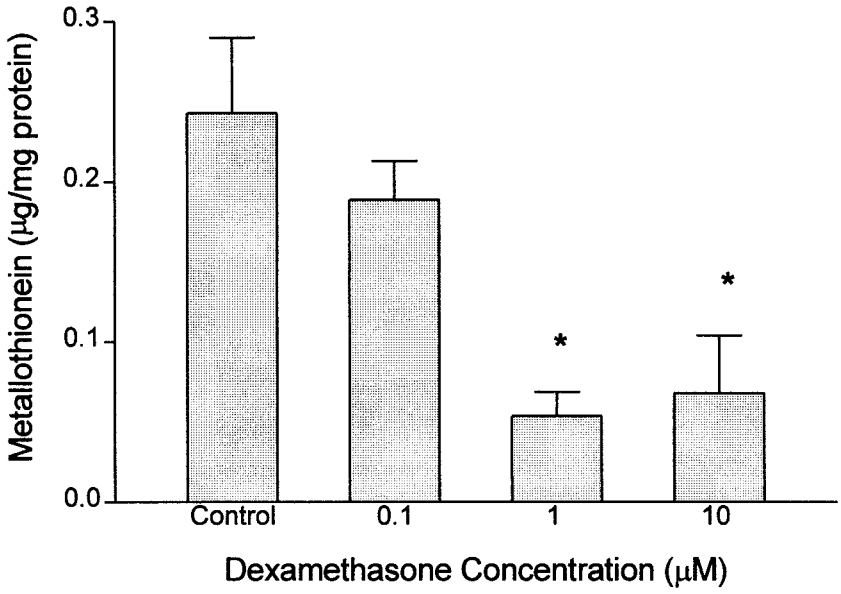

Fig. 4. Effect of dexamethasone treatment on basal levels of cellular metallothionein in chondrocyte cell cultures. For 2 days prior to metallothionein analysis, 21-day-old cultures were treated with dexamethasone at the indicated levels. Asterisks (*) indicate MT levels significantly different ($P < 0.01$) from those of the control.

(1 μM) treatment also caused an apparent increase in the levels of G-3-P dehydrogenase mRNA included in the RT-PCR reaction as an internal control.

The level of MT induction as well as basal levels of MT was dependent on the developmental state of the cell cultures (Fig. 8). During the early stages of culture the highest basal and inducible levels of MT were observed. MT levels declined steadily as the age of the cultures increased at 14–35 days.

DISCUSSION

The occurrence of metallothionein and its induction by trace metals and other agents has been studied extensively in a wide variety of

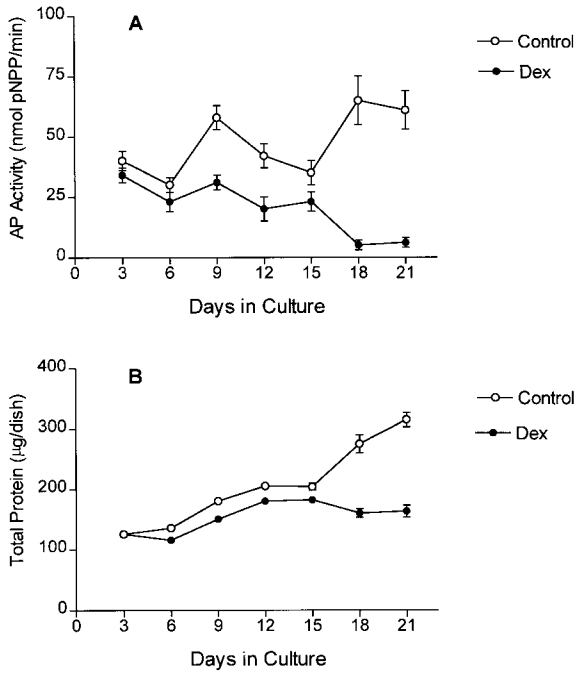

Fig. 5. Effect of dexamethasone treatment on alkaline phosphatase and cellular protein. Dexamethasone treatment (0.1 µM) was started on day 3 and continued throughout the culture period. Differences between the control and dexamethasone treatments were significant ($P < 0.01$).

organisms and tissues [Bremner and Beattie, 1990; Hamer, 1986]. Virtually all these studies have focused on liver and kidney where the highest levels of MT are observed, as well as many other soft tissues, such as brain, testes, intestine, and fish gills. The identification and induction of MT in mineralized connective tissues have received almost no direct examination. Earlier studies using embryonic bone tissue culture showed an induction of a metallothionein-like protein by Cd and Zn in cytosolic extracts of chicken femurs [Kaji et al., 1986, 1988], but the precise nature of the identified protein is uncertain. Since mineralized tissues such as bone and cartilage contain relatively high levels of trace metals [Guggenheim and Gaster, 1972] and are targets of metal ion toxicity [Wallach and Chaumser, 1990], information on the molecular mechanisms of cellular metal processing is essential to a thorough understanding of their physiology. The relative lack of information connective tissue MT likely stems from the difficulty in isolating sufficient cellular material from the dense extracellular matrices to conduct the analysis. The leg bones of young, rapidly growing chickens have elongated epiphyseal growth plates which have provided us with sufficient amounts of calcifying cartilage for this purpose. The present findings show for the first time that inducible metallothionein is present in growth plate cartilage tissue. With long-term metal exposure growth plate tissue MT levels approached those in the kidney but remain much lower than liver MT levels. The chicken growth plate MT was found to have the same physical properties as those of the single MT isoform, which has been characterized from avian liver [McCormick et al., 1988].

In primary cultures of chondrocytes isolated from the epiphyseal growth plate MT was induced by Cd >0.1 µM and Zn >100 µM. These concentrations are below the levels at which discernible toxic effects are first observed in these cells [Litchfield et al., 1997]. Previous studies have demonstrated the expression of MT mRNA in cultures of nonmineralizing mammalian articular cartilage [Zafarullah et al., 1992, 1993] and sternal cartilage [Veness-Meehan et al., 1991] chondrocytes exposed to Cd or Zn. In rat rib chondrocytes, increased expression of MT mRNA was not observed at Cd concentrations of <1 µM [von Zglinicki et al., 1992]. Induction of a metallothionein-like protein has been demonstrated in a mineralizing osteoblastic cell line [Miyahara et al., 1986] at higher Cd concentrations than we observed in primary chondrocyte cultures. Two osteoblastic cell lines, derived from mammalian osteosarcoma have been shown to have drastically different responses to Cd exposure [Angle et al., 1993]. The rat osteosarcoma line ROS 17/2.8 did not produce detectable MT in response to Cd or Zn exposure, while the human line HOS TE 85 showed a typical MT induction response. The RO 17/2.8 cells were also found to be extremely sensitive to Cd toxicity. In the present study, growth plate chondrocytes had a MT induction response to Cd comparable to what has been demonstrated in primary cultures of liver hepatocytes [Klaasen and Liu, 1991b]. In contrast, chondrocytes were approximately 10-fold less sensitive than hepatocytes to the induction of MT by Zn. Surprisingly, dexamethasone, a well known inducer of metallothionein synthesis in hepatocytes and other cells reduced MT protein levels in chondrocytes (Fig. 4), despite increased expression of MT mRNA (Fig. 7). We showed previously that other nonmetal MT inducers such as sodium butyrate and Vitamin D_3

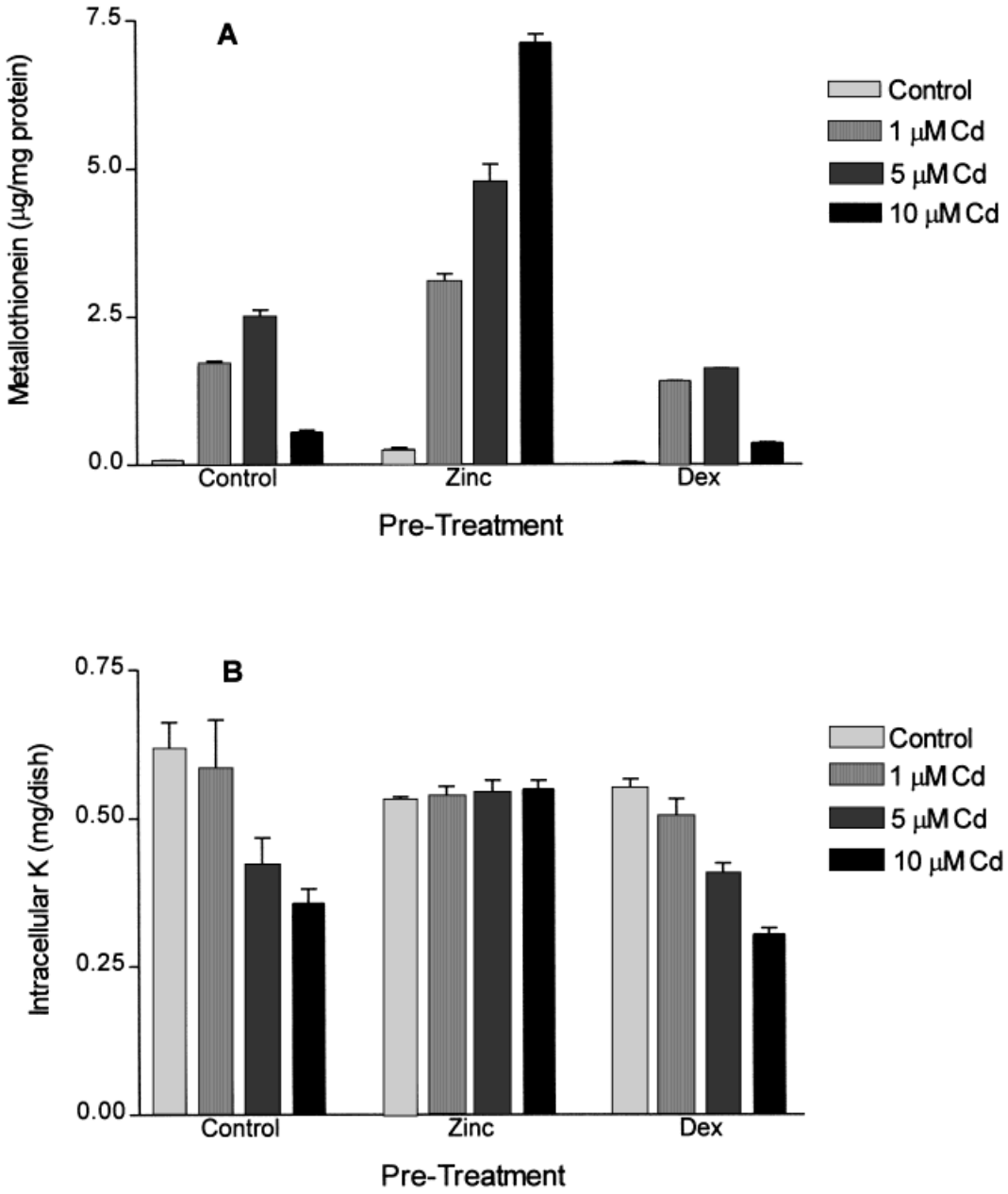

Fig. 6. Effect of 2-day pre-treatment of 20-day-old growth plate chondrocyte cultures with zinc (100 µM) or dexamethasone (1 µM) prior to cadmium exposure. **A:** Metallothionein induction was enhanced by Zn pretreatment but reduced by dexamethasone. **B:** Higher levels of metallothionein protected cells from Cd toxicity, as indicated by intracellular potassium levels.

cause low level induction of MT in cultured chondrocytes [Litchfield and Sauer, 1996].

Epiphyseal growth plate tissue contains relatively high levels of Zn, comparable to levels in liver (unpublished observations). Within the growth plate this metal has a number of specific cellular and extracellular functions related to enzyme activity and matrix mineralization [Genge et al., 1988; Sauer et al., 1989, 1994]. One role for chondrocyte MT would be to serve as an intracellular storage site for Zn^{2+} ions required for synthesis of cellular enzymes or released from the cell in matrix vesicles [Sauer and Wuthier, 1992]. From the present findings (Fig. 6), induction of chondrocyte MT also provides protection against the effects of toxic metal ions. When MT levels were reduced by dexamethasone treatment, the cells were more sen-

sitive to Cd. Elevation of cellular MT by Zn pre-treatment resulted in a greater resistance to Cd. The protective effect of MT against metal toxicity has been described in many different tissues [Hamer, 1986]. Since mineralized connective tissues are important sites for accumulation of toxic metal ions [Wallach and Chaumser, 1990], a protective role of MT in these tissues has potential physiological significance. Our observations that MT levels decline with age of the culture and the unusual effects of dexamethasone suggest that MT may have an additional role in chondrocyte cell differentiation.

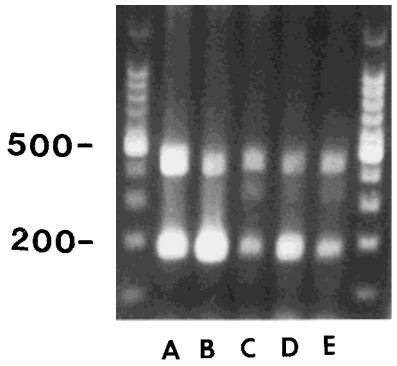

Fig. 7. RT-PCR analysis of metallothionein mRNA in chondrocyte cell cultures following 24-h exposure to inducing agents. Messenger RNA isolated from the cultures were used to make DNA templates with reverse transcriptase which were then amplified using PCR primers for a 182-bp transcribed segment of the chicken metallothionein gene. PCR products were separated on a 2% agarose gel and stained with ethidium bromide. Inducing agents were 1 μM cadmium (**A**); 100 μM zinc (**B**); 100 nM retinoic acid (**C**); 1 μM dexamethasone (**D**); none—control (**E**). DNA standards (100-bp ladder) are on outside lanes.

During the process of endochondral bone formation, chondrocytes pass through a tightly controlled series of distinct developmental phenotypes [Hunziker, 1994]. Initially, resting chondrocytes begin to proliferate forming columns of cells and increasing the length of the bone. These proliferative cells produce an extracellular matrix rich in type II collagen and proteoglycans. As the cells become surrounded by newly synthesized matrix, they enlarge and become hypertrophic. The hypertrophic chondrocytes are a distinct developmental phenotype that down-regulate type II collagen while expressing alkaline phosphatase, type X collagen, and several bone related proteins [Leboy et al., 1988; Lian et al., 1993]. As the cells become hypertrophic, they release extracellular matrix vesicles that are enriched in calcium, inorganic phosphate, and Zn [Sauer et al., 1989]. The matrix vesicles are the initial sites of calcium phosphate mineral formation thereby triggering matrix calcification [Wuthier, 1988]. Recent findings indicate that as the matrix calcifies the entrapped hypertrophic cells enter a terminally differentiated stage characterized by apoptotic cell death [Gibson et al., 1995; Hatori et al., 1995]. The chondrocyte culture system used in the present study parallels morphologically and biochemically the changes that occur in vivo [Wu et al., 1995]. Upon attaining confluency

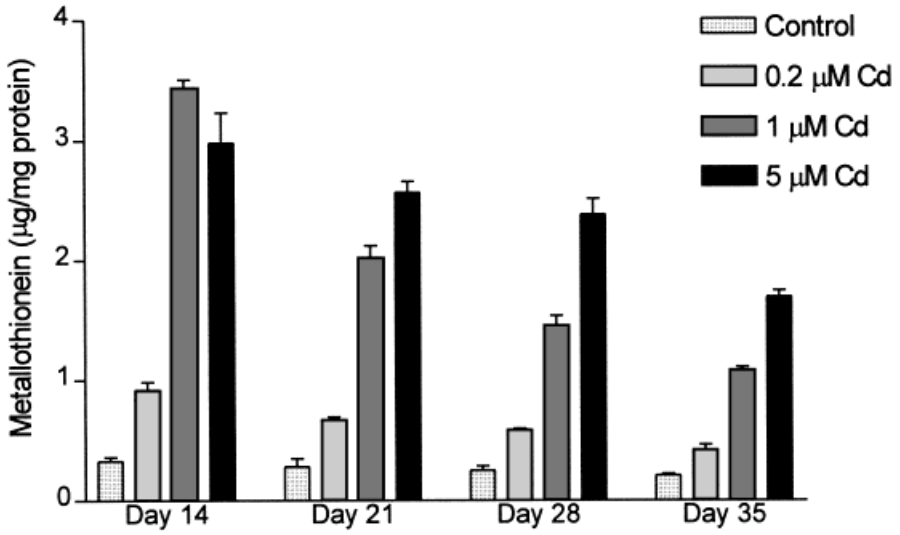

Fig. 8. Induction of metallothionein by cadmium at different stages of growth plate chondrocyte cell culture. Cultures at each age indicated were exposed to Cd for 2 days prior to analysis for metallothionein. Declines in the levels of basal and inducible metallothionein from one time period to the next were statistically significant ($P < 0.05$).

from days 10–14, the cells begin to proliferate multicellular nodules. Within these nodules, cells become hypertrophic and begin to release calcifiable matrix vesicles between days 21–28, and by day 35 the cultures are fully mineralized. Our present findings show that as the cultures progress through the different developmental stages, the basal and inducible levels of MT decline (Fig. 8). This may be due to the effects of intracellular reactive oxygen species (ROS) generated by terminally differentiated chondrocytes as they become apoptotic. ROS production is a common feature of apoptotic cells and is thought to contribute to the final degradative stages of programmed cell death that includes nuclear disintegration and cytolysis [Kroemer et al., 1995]. Chondrocytes are know to generate large amounts of ROS such as nitric oxide under certain conditions [Evans et al., 1996]. Studies have shown that the metal–thiolate bonds of metallothionein are highly sensitive to oxidation by ROS causing the release of the metal ion [Fliss and Menard, 1992]. The oxidative release of metal ions from MT and other intracellular metalloproteins in apoptotic chondrocytes may account for the elevated levels Zn^{2+} that occur in matrix vesicles [Sauer et al., 1989]. A similar process could contribute to the incorporation of toxic metal ions such as Cd into calcified cartilage and bone matrix.

Dexamethasone induces the synthesis of MT in vertebrate cells by its interaction with the glucorticoid response element in the promoter region of the MT gene [Hamer, 1986]. Dexamethasone has also been shown to influence chondrocyte differentiation, blocking cell proliferation and promoting progression to the hypertrophic terminally differentiated phenotype [Quarto et al., 1992; Yasuda et al., 1995]. Consistent with this effect is a decline in the specific activity of alkaline phosphatase, as we have seen in the later stages of chondrocyte culture [Wu et al., 1995] and in response to dexamethasone supplementation (Fig. 5). It thus appears likely, that the decline in MT levels we have observed in response to dexamethasone treatment is the result of increased intracellular degradation of MT associated with chondrocyte apoptosis, rather than a down-regulation of MT gene expression. The specific controls of MT gene expression in differentiating chondrocytes, as well as the mechanisms of cytosolic degradation of MT need to be clarified with further study.

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