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Inhibition of Induced Endochondral Bone Development in Caffeine-Treated Rats

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Abstract We have addressed questions raised by the observation in fetal rats of delayed ossification induced by caffeine at maternal doses above 80 mg/kg body weight per day. The effect of caffeine on endochondral bone development and mineralization has been studied in an experimental model system of bone formation which involves implantation of demineralized bone particles (DBP) in subcutaneous pockets of young growing rats. Caffeine's effects on cellular events associated with endochondral ossification were examined directly by quantitating cellular mRNA levels of chondrocyte and osteoblast growth and differentiation markers in DBP implants from caffeine-treated rats harvested at specific stages of development (day 7 through day 15). Oral caffeine administration to rats implanted with DBP resulted in a dose dependent inhibition of the formation of cartilage tissue in the implants. Histologic examination of the implants revealed a decrease in the number of cells which were transformed to chondrocytes compared to control implants. Those cartilaginous areas that did form, however, proceeded through the normal sequelae of calcified cartilage and bone formation. At the 100 mg/kg dose, cellular levels of mRNA for histone, collagen type II, and TGFβ were all reduced by greater than 40% of control implants consistent with the histological findings. Alkaline phosphatase activity in the implants and mRNA levels for proteins reflecting the hypertrophic chondrocyte and bone phenotype, collagen type I and osteocalcin were markedly decreased compared to controls. Lower doses of 50 and 12.5 mg/kg caffeine also resulted in decreased cellular proliferation and transformation to cartilage histologically and reflected by significant inhibition of type II collagen mRNA levels (day 7). The effects of caffeine on gene expression observed in vivo during the period of bone formation (day 11 to day 15) in the DBP model were similar to the inhibited expression of H4, alkaline phosphatase, osteocalcin, and osteopontin found in fetal rat calvarial derived osteoblast cultures following 24 hour exposure of the cultures to 0.4 mM caffeine. Thus the observed delayed mineralization in the fetal skeleton associated with caffeine appears to be related to an inhibition of endochondral bone formation at the early stages of proliferation of undifferentiated mesenchymal cells to cartilage specific cells as well as at later stages of bone formation.

Key words: caffeine, bone matrix implants, delayed ossification, osteoblasts, gene expression

In recent years, studies have shown that caffeine alters the normal processes of skeletal mineralization and development in animals. The effects on the fetal skeleton are well documented [Fuji and Nishimura, 1972; Palm et al., 1978; Nolen, 1981; Collins et al., 1983]. Maternal caffeine ingestion of 20 mg/kg body weight or more produced a dose dependent increase in the incidence of unossified sterna, misshapen or missing vertebral centra and reduced ossification of the metacarpals and metatarsals [Collins et al., 1983; Nolen, 1981; Scott, 1983]. Fetal bone development and endochondral bone formation is a complex process regulated by hormones and local factors affecting bone cell replication and differentiated functions. The process of endochondral ossification occurs normally in the developing fetal skeleton and is the process by which elongation of bone occurs throughout development and growth. The sequential, programmed events of endochondral bone formation from mesenchymal differentiation to chondrocytes with subsequent cartilage forma-

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tion and calcification, resorption and replacement by bone forming cells can be evaluated in a model system. This system, which utilizes demineralized bone particles (DBP) to serve as inductants of bone formation in non-osseous sites, has been well characterized [Urist, 1965; Reddi and Huggins, 1972; Reddi and Anderson, 1976; Glowacki and Mulliken, 1985].

This model was characterized for expression of selected cell growth-regulated and tissue-specific genes during cartilage and bone formation [Bortell et al., 1990; Carrington et al., 1988]. The studies correlated well with the histological and biochemical events documented by earlier investigators [Reddi and Huggins, 1972] and allow for a greater level of analysis of the events leading to the proliferation, differentiation, and mineralization of endochondral bone. The linear, reproducible series of events in this DBP model system make it valuable for the evaluation of exogenous compounds on bone formation [Glowacki, 1982]. This paper reports the effects of caffeine on the events associated with endochondral bone formation using this DBP model. We addressed whether the skeletal malformations observed in fetal rats induced by maternal caffeine ingestion were a consequence of a generalized teratogenic effect or a more direct effect on cartilage or bone development. The present studies demonstrate oral caffeine ingestion results in a dose related inhibition of cartilage and bone induction as observed histologically and by decreases in cell proliferation (histone and c-myc mRNA), cell growth (TGFβ mRNA), alkaline phosphatase activity, collagens type II and type I, and osteocalcin mRNA levels. The observed in vivo effects of caffeine on bone formation were also compared to expression of osteoblast growth and differentiation related genes in an in vitro model of bone formation using fetal rat calvarial derived osteoblasts [Aronow et al., 1989; Owen et al., 1990]. Cultures treated for 24 h with caffeine (0.4 mM) showed decreased levels of histone H2B, osteopontin, osteocalcin and alkaline phosphatase throughout the stages of osteoblast phenotype development.

METHODS

Studies were carried out in a series of two experiments. In the first, the level of caffeine exposure was 100 mg/kg/day. Results from these experiments provided the basis for the second set of experiments in which lower doses of caffeine (as detailed below) were employed to determine whether the effects noted at the high (100 mg) dose were present in a dose related manner.

Animals

For all experiments, male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were obtained at 21 days of age. The rats were randomly assigned to either an experimental group which received caffeine (Sigma Chemical Co., St. Louis, MO) in their drinking water or to a control group receiving distilled water. The caffeine was dissolved in distilled water at concentrations calculated to deliver 100 mg/kg/day. For some experiments solutions of caffeine were made to deliver either 12.5 mg/kg or 50 mg/kg per day. The animals received fresh solutions 3× per week and water intake was measured at that time. When necessary, caffeine concentrations in the drinking water were adjusted to allow for the growth of the animals and increased water intake in the high dose caffeine treated groups. The animals were weighed twice weekly and at the time of explantation. Seven days after the initiation of caffeine exposure, (28 days of age) the rats received two implants of demineralized bone particles placed into subcutaneous pockets as described below. The rats continued to receive caffeine throughout the implantation period. Groups of n = 6 animals (12) per experimental time point were evaluated, using n = 4 implants per assay. For comparative analysis of various assays, one implant of each rat was used for histologic analysis while the other was designated for biochemical assays.

Preparation of Demineralized Bone Particles (DBP)

Bone powder was prepared from the diaphyses of tibia and femurs of 6–10-week-old rats. The cleaned diaphyses were extracted with absolute ethanol followed by anhydrous ether, dried then pulverized in a Spex liquid nitrogen mill and sieved to particle sizes of 75–250 μm. Demineralization of the bone powder was accomplished by extraction with 0.5 M HCl (25 meq/gm bone) for three hours at room temperature followed by washes in distilled water to remove all acid, absolute ethanol, and ether and lyophilized as described by Glowacki and Mulliken [1985].

Implantation Procedure

Male, day 28, Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were im-

planted with 50 mg of demineralized bone particle prepared as described above. The DBP was implanted in two subcutaneous pockets located bilaterally on the thoracic region in the rats [Reddi and Huggins, 1972]. Within 24 h, the DBP became encapsulated forming a discrete implant. The bone implants were removed from the rats over a time course spanning 7 to 15 days after implantation at time points selected to demonstrate specific stages of the endochondral bone sequence.

Rat Osteoblast Cultures

Normal diploid osteoblast from gestational day 21 rat calvaria were obtained from sequential trypsin-collagenase digestions and placed at $6.4 \times 10^3/cm^2$ in MEM with 10% fetal bovine serum (FBS) [Aronow et al., 1989; Owen et al., 1990]. At confluence (day 7–8), the media was changed to BGJ_b with 10% FBS, 50 mg/ml ascorbic acid, and 10 mM β-glycerophosphate. Beginning on day 6, the cells were exposed to fresh media containing either 0 mM or 0.4 mM caffeine for 24 h on days 6, 10, 13, 17, and 25. At each time point, cells were harvested for measurement of alkaline phosphatase and analysis of mRNA transcripts from the genes listed.

Histological Analysis

Explants from each animal were fixed in 2.5% paraformaldehyde. Specimens were dehydrated and sectioned in JB4 embedding media (Polysciences), for evaluation of cartilage by Toluidine blue stain, alkaline phosphatase activity using Sigma reagents (Sigma Chemical Co., St. Louis, MO), and for mineral by 3% silver nitrate (Von Kossa stain).

RNA Isolation and Analysis

The bone implant was excised from the rats and immediately immersed in liquid nitrogen. The specimen was then pulverized under liquid nitrogen. Total cellular RNA was isolated from each cell pellet and DBP implant powder by the SDS-proteinase K method as described by Plumb et al. [1983]. RNA preparations were quantitated by absorbance at 260 nm. Intactness was assessed by ethidium bromide staining following separation in 6.6% formaldehyde-1% agarose gels. RNA fractionated in such gels was transferred to Zeta-Probe membrane (BioRad, Richmond, CA) in 20 × SSC (0.3M sodium chloride, 0.3M sodium citrate) by the capillary method of Thomas [1983]. In other cases, RNA samples were bound to Zeta-Probe blotting membrane by slot blot under conditions described by the apparatus manufacturer (Schleicher and Schuell, Keene, NH). DNA probes used for hybridization were the following: rat H4 histone [Grimes et al., 1987]; c-myc [Toscani et al., 1988]; rat Type I collagen [Genovese et al., 1984]; rat Type II collagen [Kohno et al., 1984]; rat osteopontin [Oldberg et al., 1986]; rat osteocalcin [Lian et al., 1989]; and human transforming growth factor β (TGF-β) [Derynck et al., 1985]. All DNA probes were labeled with [^{32}P]dCTP by the random primer method [Feinberg and Vogelstein, 1983]. For matrix Gla protein (MGP) a synthetic oligonucleotide probe spanning nucleotides 256–258 [Price et al., 1987] was labeled with [V^{32}P] dATP at the 5′ end using T4 polynucleotide kinase. Prehybridizations and hybridizations were performed at 42°C in 50% formamide; 5× SSC; 5× Denhardt's; 50 mM PO_4 buffer (pH 6.5); 1% SDS; 250 g/ml salmon sperm DNA at 42°C for 4 h. For hybridizations. 10^6 cpm/ml probe and 10% dextran sulfate were added [Maniatis et al., 1982]. Blots were washed twice at room temperature and once at 65°C in 2× SSC/0.1% SDS (15 min per wash) and then twice at 65°C in 0.1× SSC/0.1% SDS (30 min per wash). Autoradiographs were quantitated by scanning laser densitometry and values normalized to 28S ribosomal RNA.

Biochemical Analysis

Explants were evaluated for alkaline phosphatase activity after homogenization of the implant in 0.1% MgCl glycine buffer, using p-nitrophenol phosphate as substrate. Serum from control and caffeine-treated rats was obtained by cardiac puncture prior to sacrifice and assayed for osteocalcin by radioimmunoassay [Lian and Gundberg, 1988].

RESULTS

In Vivo Studies, Morphology

When demineralized bone particles (DBP) are subcutaneously implanted into growing rats the implants exhibit a tightly controlled progression of events which occur in endochondral bone development. The time points in the present study were selected to illustrate the progressive stages of endochondral bone formation in the DBP implants. Day 7 corresponds to the formation of cartilage which calcifies by day 9. On day

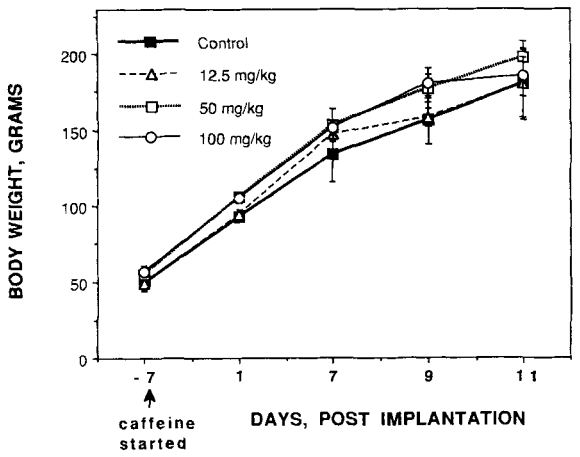

Fig. 1. Effects of caffeine on the growth of the rats. Body weight (g) of the animals receiving 0, 12.5, 50, and 100 mg/kg of caffeine in their water were measured regularly during the experiment.

11, bone is formed, and by day 15, bone formation is complete and the onset of bone remodeling is observed. The dose of caffeine chosen, 100 mg/kg, was in the range which has been reported to induce delayed ossification in the fetuses of treated dams. Subsequently, doses were chosen to determine whether there was a dose dependent effect of caffeine on endochondral bone formation.

At all doses growth of the rats, as measured by body weight, did not vary from control animals (Fig. 1). Water consumption measured as ml/rat/day was unchanged in the first experiment and in the second, except for the 50 mg/kg dose group. These animals consumed slightly greater volumes of water beginning from postimplantation day 4 through the end of the study. For example, on day 4, controls consumed 19.4 ± 2.9 vs. 24.8 ± 1.4 mls/rat/day on the 50 mg dose. This observation is consistent with other studies that have shown that caffeine intake will increase fluid and food consumption [Palm et al., 1978; Nolen, 1981]. The effect of caffeine on bone turnover in the rats was evaluated by serum osteocalcin levels. While the levels decreased as a function of age of the rats from 190 ± 16 (day 7 post-implantation) to 155 ± 4 ng/ml (day 14 post-implantation) for control, no significant difference was found in the caffeine treated group (day 7, 167 ± 15; day 14, 141 ± 21, $n = 6, P < .05$).

Histologic examination of the implants in the 100 mg/kg experiments revealed marked differences in cartilage formation in the implants beginning on day 7. Implants stained with toluidine blue showed numerous foci of chondrocytes in controls while barely detectable areas of cartilage differentiation were found in implants from caffeine-treated animals (data not shown). At 9 days, the implants from control animals were mostly cartilaginous throughout (Fig. 2A,B), in contrast to the implants from caffeine treated animals (Fig. 2D,E) in which the few areas present on day 7 increased only slightly in size by day 9 and new areas of chondrogenesis were not apparent. On day 9, the caffeine exposed implants exhibited only weakly positive staining for alkaline phosphatase activity in cartilage areas, unlike day 9 control implants, where alkaline phosphate activity was present throughout (Fig. 2C,F). Biochemically, alkaline phosphatase activity measured in the entire implant was 55% of control values in caffeine treated animals on days 9 and 11 (Table I). Mineralized matrix associated with hypertrophic chondrocytes was observed at day 9 in control implants (Fig. 3A,B), but no evidence of calcification was found in implants from caffeine-treated rats (Fig. 3C,D). By day 11, bone formation had begun in the areas of calcified cartilage in the control implants (Fig. 4A–C). In caffeine exposed implants (Fig. 4D–F), no new areas of cartilage had formed but those that were present on day 9 had calcified and appeared to be undergoing a normal sequence of conversion to bone.

In Vivo Studies, Gene Expression

Our results show that mRNA levels (Fig. 5) from the control implants correlate very well with the known developmental sequence of protein expression in this system [Reddi, 1981]. For example as shown for the controls, mRNA levels for H4 histone and collagen type II, a phenotypic marker of cartilage, are highest at 7 days of implantation, when proliferation of chondrocytes is occurring. Matrix Gla protein, abundant in developing cartilage, is expressed at highest levels during the chondrogenic period (days 7–9) [Hale et al., 1988; Barone et al., 1991]. Type I collagen, TGFβ, and c-myc expression are highest during the stage of calcified cartilage formation and the onset of osteoblast activity and bone matrix formation. Since Figure 5 illustrates levels of gene expression as % maximal for comparison of control to caffeine-treated groups for each gene, the relative abundance of cellular mRNA representation for each gene in control implants is shown in Figure 6. Here it is appreciated that osteocalcin mRNA, an osteoblast

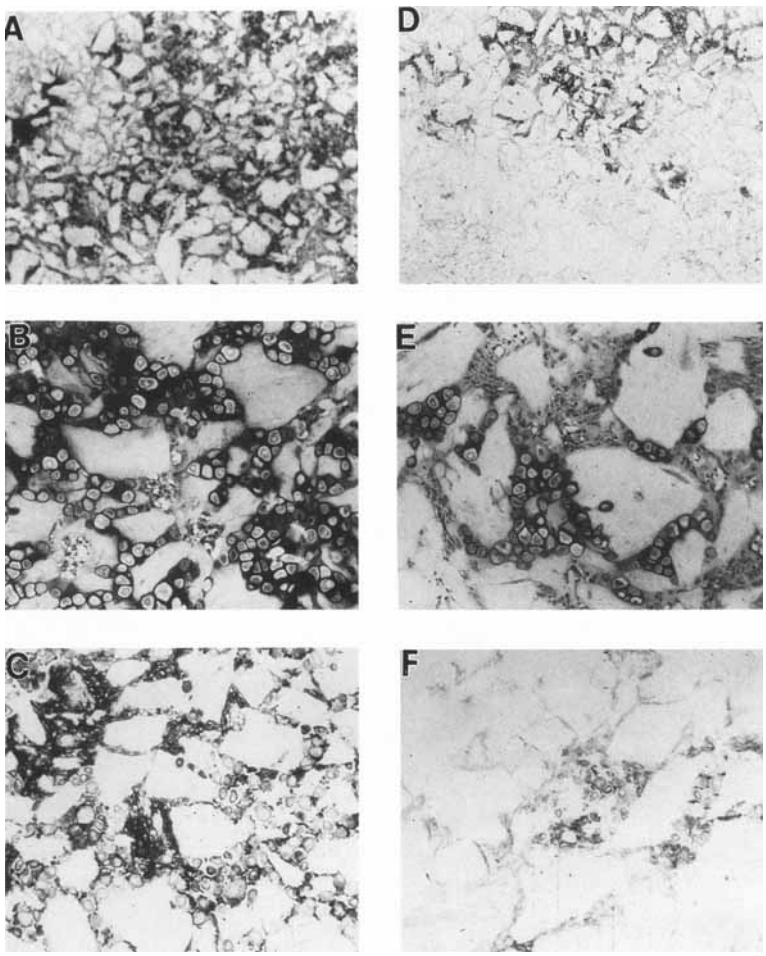

Fig. 2. Histologic sections of DBP implants during cartilage formation Day 9 implants were stained with toluidine blue from control animals (**A**, ×100, **B**, ×320) and caffeine treated rats (**D**, ×100, **E**, ×320) Low magnification (A,D) illustrates decreased area of chondrogenesis in implants from caffeine treated rats Higher magnification (B,E) shows chondrocytes surrounding DBP Undifferentiated mesenchymal cells are found throughout the caffeine affected implants Implants stained for alkaline phosphatase and with toluidine blue between (control [**C**] and caffeine [**F**] at 100×) also shows differences in amount of chondrogenic tissue

TABLE I. Alkaline Phosphatase Activity in Control and Caffeine Treated Rats*

Day	Control (nm/mg implant)	Caffeine (nm/mg implant)
7	24 43 ± 2 73	30 35 ± 15 92
9	87 62 ± 16 68	41 26 ± 4 84
11	127 43 ± 21 52	69 21 ± 35 92

*Alkaline phosphatase activity in nanomoles of p-nitrophenol/mg of implant Values are mean ± S D for n = 4 implants ($P < 01$, days 9 and 11) Significant differences occur in days 9 and 11 implants

marker, is one of the lowest represented bone matrix related mRNA's compared to the collagens. It was detected on day 7 at trace levels and began to increase on day 15 when mineralized bone matrix was forming throughout the implant. To allow for variations in the amount of total RNA between treatments, 28S ribosomal RNA was selected as an internal standard. This did not change and therefore was utilized for normalization. Taken together, these data suggest that mRNA levels represent a convenient and reliable indicator of developmental changes in the endochondral bone development system.

Caffeine had profound effects on the mRNA levels of genes related to both proliferation and the phenotypic expression of normal bone (Fig. 5). The mRNA levels for cell proliferation (histone H4) were decreased 20% from control by caffeine treatment early in the time course (day 7), but showed a 50% reduction by day 11 when compared to control. The c-myc oncogene mRNA, also associated with growth as well as differenti-

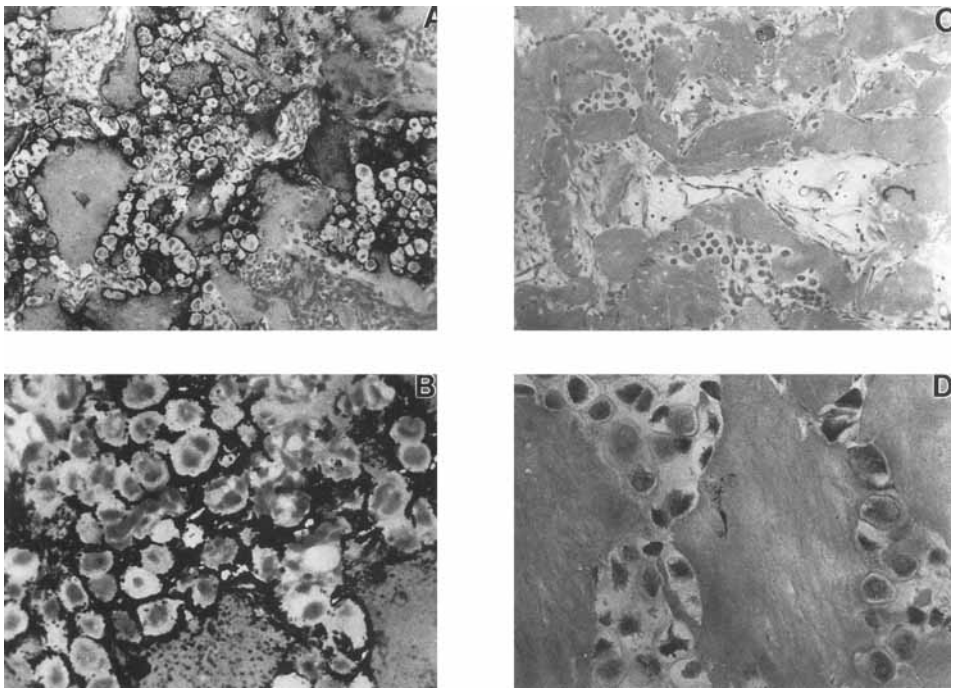

Fig. 3. Detection of mineralization in histologic sections of DBP implants during cartilage matrix calcification Day 9 implants were stained with von Kossa silver stain and counter stained with hematoxylin and eosin Implants from control rats (**A**, ×100, **B**, ×320) show hypertrophic chondrocytes and extensive matrix mineralization compared to absence of mineral in cartilage areas of implants from caffeine treated rats (**C**, ×100, **D**, ×320)

ation, was reduced 2–4-fold in caffeine treated rats. Transforming growth factor β (TGF-β) is associated both with proliferation and the induction of the extracellular matrix. mRNA levels of this gene were also 40–44% decreased with caffeine treatment. Strikingly, collagen type II and MGP expression, markers of chondrogenesis and early stage of bone development in this system, were profoundly decreased (up to 10-fold) in caffeine treated rats at days 7 and 9. Collagen type I mRNA levels were reduced by 2–3-fold throughout the stages of bone development. While levels remained significantly lower in the caffeine group, controls exhibited an increase. Osteocalcin levels (data not shown) were 50% lower than controls on day 15, the peak of bone formation. These results suggest a delay in progression to bone tissue as indicated histologically (Figs. 2–4).

At lower doses of caffeine (12.5 and 50 mg/kg), decreased transformation of recruited mesenchymal cells to chondrocytes was also observed but to a lesser extent than the 100 mg/kg dose (data not shown). The quantitative decrease in histone and collagen Type II mRNA (Fig. 7) to 75% of control values reflects the decrease in cellular proliferation and cartilage formation observed morphologically.

In Vitro Experiments

Previous studies in our laboratory [Tassinari et al., 1991] have shown that chronic caffeine exposure to normal diploid chick-derived osteoblasts in culture had direct effects on osteoblast activity and bone matrix formation. Formation of a mature extracellular matrix and subsequent mineralization of that matrix was reduced with 0.4 mM caffeine exposure but not with 0.1 and 0.2 mM doses. These changes were reflected by decreased collagen accumulation in the matrix, inhibition of alkaline phosphatase activity, and decreased osteocalcin synthesis. No analysis for gene expression was carried out. In the present study, acute caffeine exposure (24 h, 0.4 mM) was examined in a fetal rat calvarial osteoblast culture system at specific stages of the development of the osteoblast phenotype. The progression of isolated normal osteoblasts through a developmental sequence of cellular differentiation is dependent upon formation of mineralized bone-like matrix and is characterized by the temporal expression of genes which define devel-

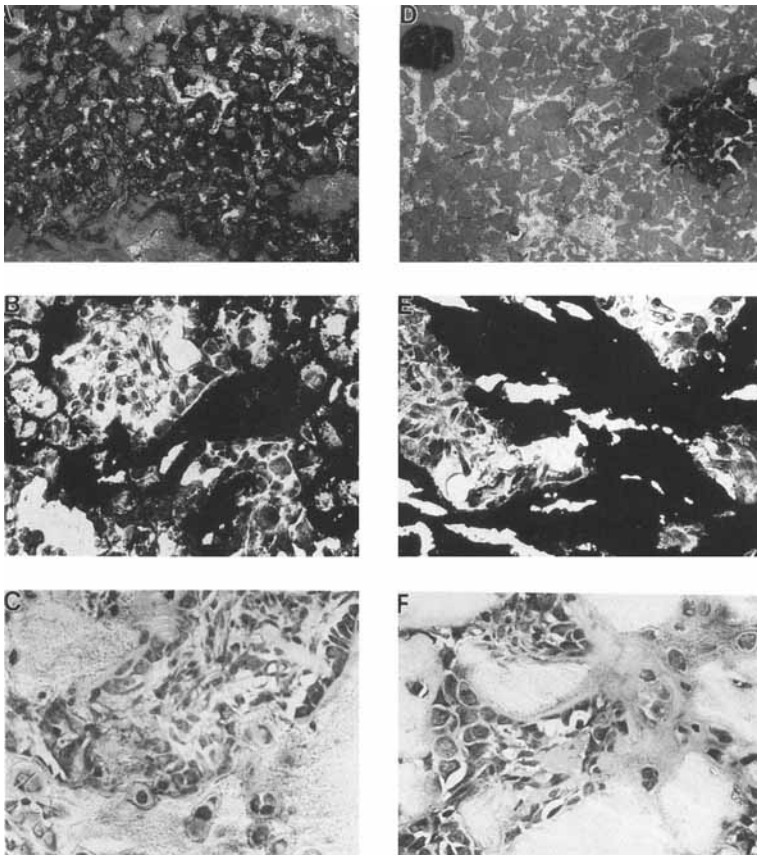

Fig. 4. Histologic sections of DBP implants from control and caffeine treated rats during bone formation Day 11 implants were stained for mineral with von Kossa silver stain and counter stained with hematoxylin and eosin (control, **A**, caffeine, **D**, both at ×100) Higher magnifications are shown for implants stained with von Kossa silver stain and counterstained with toluidine blue (control, **B**, caffeine, **E**, ×320) In lower panels, cellularity is visualized with toluidine blue staining (control, **C**, caffeine, **F**, ×320)

opmental periods of proliferation, matrix maturation and mineralization [Owen et al., 1990].

Cultures of rat osteoblasts were exposed to 0 or 0.4 mM caffeine acutely (24 h) and assayed on the days indicated in Figure 8. Depending on the stage of the osteoblast maturation, acute exposure to caffeine had differential effects on the expression of osteoblast genes necessary for bone formation. Early during the proliferative period (day 7), expression of cell growth genes, histone H2B and osteopontin (2ar), were greatly inhibited with caffeine treatment; in contrast, cellular levels of collagen mRNA were increased. Effects on these parameters after completion of the proliferative period were less pronounced during the period of matrix maturation and onset of mineralization (days 14–18). Osteocalcin, however, was significantly inhibited (50%) in caffeine-treated cultures. Alkaline phosphatase enzyme activity (Fig. 9) and osteopontin mRNA levels were modestly inhibited (25%) only on day 18. In the very mature mineralized cultures (day 26), alkaline phosphatase activity and osteopontin mRNA levels were significantly reduced by 50% of controls. At this time, osteocalcin mRNA levels were down regulated and caffeine treatment had no effect. These observations indicate that the stage of osteoblast phenotype development and level of expression of a particular gene is a contributing factor to the effect of caffeine.

DISCUSSION

Caffeine appears to target the proliferative stages of tissue development, possibly inhibiting recruitment of the mesenchymal cells to the implant, but clearly inhibiting growth and differentiation of chondroprogenitor cells as shown in the histologic sections and by inhibited expression of cell growth and phenotypic related genes. In implants from caffeine-treated rats, mineralization of calcified cartilage was delayed in those

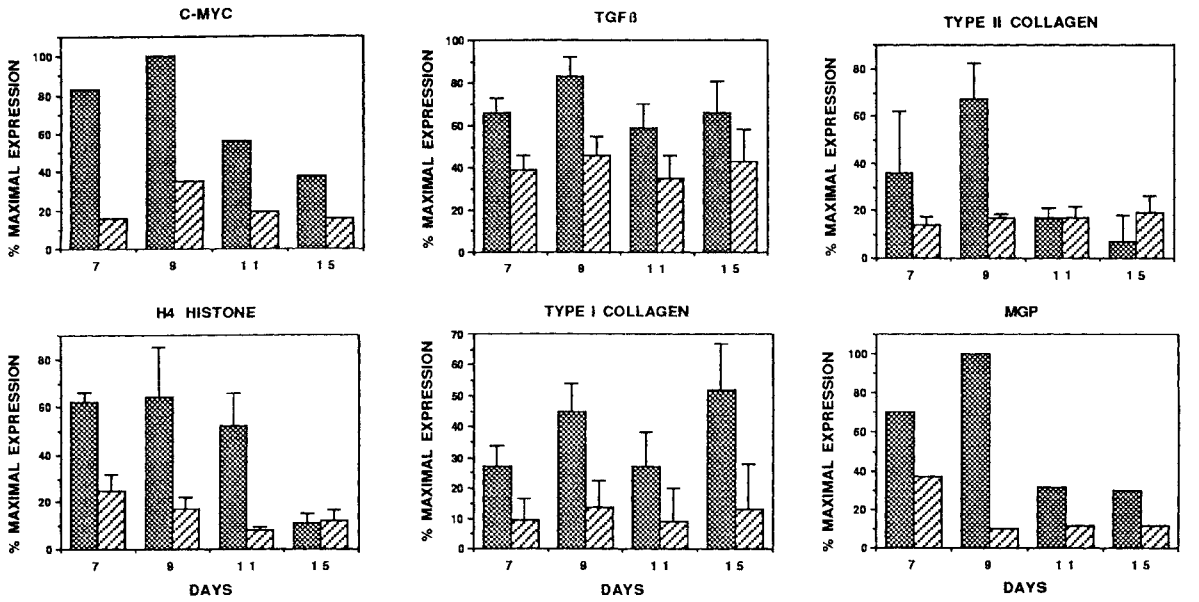

Fig. 5. Cellular mRNA expression of growth and differentiation genes during endochondral bone formation from control and caffeine treated rats (100 mg/kg). The demineralized bone particle implants were harvested at specific stages of endochondral ossification to encompass the transition from cartilage to bone. Total RNA was assayed for c-myc, H4 histone, TGF-β, and Type I and Type II collagens and MGP by Northern blot and slot blot analysis. The resulting blots were quantitated by scanning densitometry and plotted. Values are expressed as % maximal average densitometric value for the entire experimental period (days 7–15) for each parameter from duplicate blots of the same study. Error bars indicate mean ± S.E. values for n = 4 implants.

few areas where chondrogenesis was evident. The integrity of the endochondral formation sequence was, however, maintained since subsequent bone formation was observed, but only in the areas where chondrogenesis was evident. These findings suggest that the inhibition of ossification in bones, as observed in fetal sternum of rats exposed to caffeine [Nolen, 1981; Scott, 1983; Collins et al., 1983], may result from interruptions not only during mineralization of the bone but also at the earlier point of inhibition of proliferation and differentiation of chondroprogenitor cells as observed in the DBP implant model. The demineralized bone particle system is extremely sensitive to caffeine at initial stages of induction from recruitment of the fibroprogenitor cells to chondrogenic events as indicated by the effects that lower doses of caffeine had on the expression of the proliferation specific histone H2b and the cartilage specific Type II collagen gene in this model system.

These findings provide some understanding to the observations of maternal caffeine ingestion on in utero fetal rat skeletal development. Maternal caffeine ingestion of 20 mg/kg body weight of caffeine increases the incidence of unossified sterna, misshapen or missing vertebral centra and reduced ossification of the metacarpals and metatarsals [Nolen, 1981; Scott, 1983; Collins et al., 1983]. Subsequent studies in neonatal rats have shown that the delayed mineralization in the skeleton is resolved postnatally once the offspring is no longer subject to caffeine exposure [Collins et al., 1983]. Neonatal skeletons examined on postnatal day 4, showed no difference in mineralization to unexposed controls [Dews et al., 1984], although Collins et al. [1983] noted a persistence of delayed mineralization on postnatal day 6 at their highest dose (71 mg/kg). Since endochondral bone formation is a continuous process throughout growth, the removal of caffeine would permit cellular proliferation and chondrogenesis with subsequent bone formation.

In vitro, caffeine and other methylated xanthines have the ability to change the normal mode of DNA replication, inhibit cell proliferation, and inhibit active adenosine receptors [Cleaver, 1969; Fredholm, 1980; Snyder et al., 1981]. Adenosine receptors have been implicated in osteoblast activity [Rodan and Martin, 1981]. It was of interest, therefore, to observe that the effects of caffeine on the rat derived osteoblast cultures were a function of their devel-

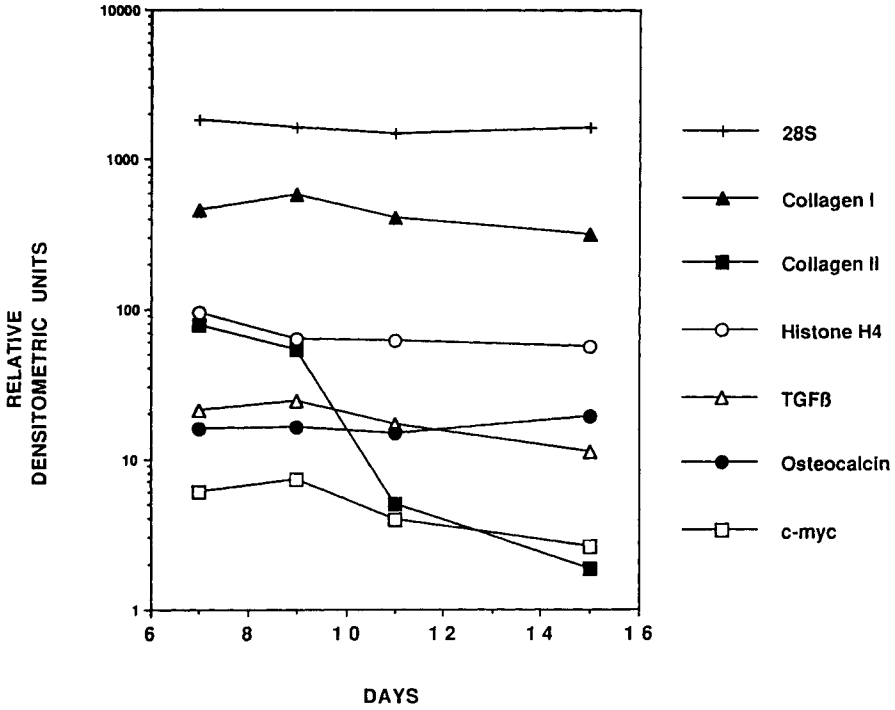

Fig. 6. Comparison of relative levels of mRNA present in control implants selected at specific points during endochondral bone formation. Relative densitometric units (y-axis) are equivalent to densitometry units/(μg RNA × h of exposure [autoradiography] × specific activity of probe × probe size).

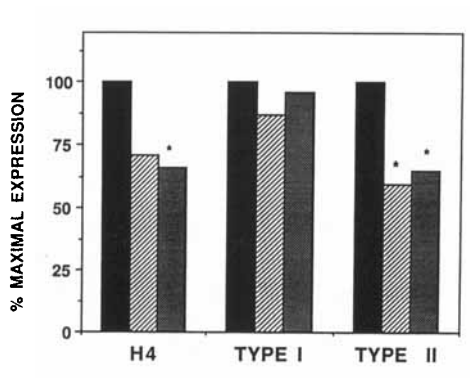

Fig. 7. Effects of caffeine (0, black bar; 12.5, hatched; and 50, shaded, mg/kg) on cellular mRNA levels of growth and differentiation genes during cartilage formation using the demineralized bone particle system. RNA was extracted from demineralized bone particle implants removed from rats at day 7 after implantation; time point corresponding to cartilage formation during the endochondral bone sequence. Prior to explantation, these rats were administered 0, 12.5, or 50 mg/kg of caffeine. RNA was assayed for H4, Type I collagen, and Type II collagen by slot blot analysis and blots were quantitated by scanning densitometry. Results are the mean of the data from three animals per time point. Asterisk indicates significance of $P < .05$, n = 4 implants compared to controls by *t*-test analysis.

opmental stages of differentiation. Actively proliferating pre-osteoblast like cells (day 6) were extremely sensitive to caffeine exposure as demonstrated by an 80% reduction in proliferation and an 80% increase in Type I collagen expression. A reciprocal increase in collagen gene expression with the cessation of proliferation has been observed in other cell types [Stepp et al., 1986]. However, on days 11 and 14, when nonproliferative cells have developed the osteoblast phenotype, that is, high collagen Type I, alkaline phosphatase activity, and osteocalcin expression, only osteocalcin mRNA is reduced significantly (50% control) in caffeine treated cultures. Then, at the late state of osteogenesis in the mineralization period, alkaline phosphatase and osteopontin were significantly inhibited in mature osteoblasts. These results indicated that within a particular stage of osteoblast phenotype development, caffeine selectively targets genes that are expressed, resulting in differential effects on specific parameters of the osteoblast. Furthermore, these acute effects (within 24 h), indicate a more direct inhibition of osteoblast related gene expression and support our observations in chick cultures exposed to chronic

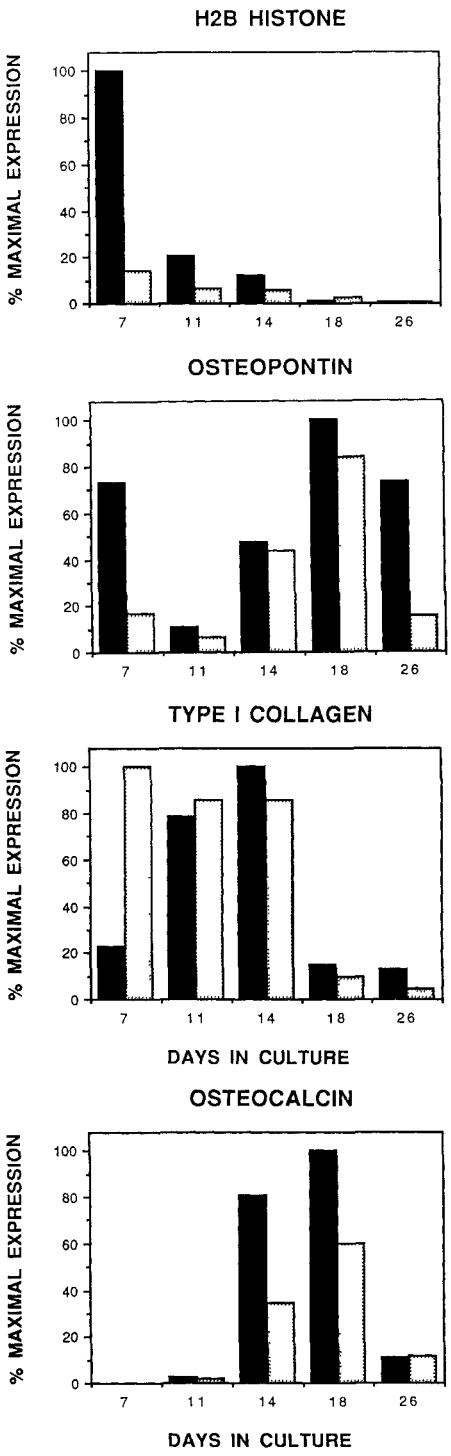

Fig. 8. Effect of caffeine on the expression of osteoblast genes using fetal rat calvarial derived osteoblast cultures. Osteoblast cultures were exposed to 0 mM (black bar) or treated with 0 4 mM caffeine (dotted bar) 24 h prior to the indicated day and then assayed for the steady state levels of H2B histone, Type I collagen, osteopontin and osteocalcin by Northern Blot analysis or slot blot analysis The blots were quantitated by scanning densitometry and the results plotted relative to the maximal expression of each mRNA transcript.

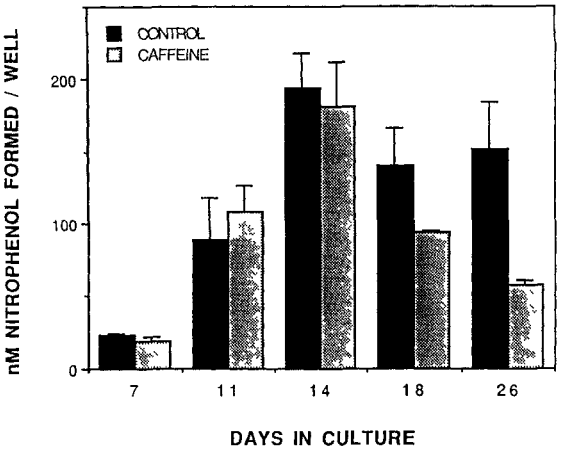

Fig. 9. Effect of caffeine on alkaline phosphatase activity in fetal rat calvarial derived osteoblasts. Osteoblast cultures were exposed to 0.4 mM caffeine for 24 h and assayed for alkaline phosphatase activity and compared to control (untreated) osteoblasts. Control cultures are represented by black bars and caffeine treated cultures as dotted bars.

caffeine. In those studies [Tassinari et al., 1991], caffeine added to cultures at confluency (day 7) resulted in a dose dependent inhibition in the ability of the osteoblasts to form an organized extracellular matrix capable of undergoing mineralization as measured by decreases in the levels of osteocalcin, alkaline phosphatase activity, and total calcium and absence of positive histochemical mineral staining.

The doses of caffeine in these in vivo and in vitro studies were much greater than the reported human equivalent intake of caffeine [Stavric, 1988]. Dosages of this order, however, in rats have been found to affect calcium intestinal absorption and stimulate an increase in $1,25(OH)_2$-vitamin D production. Caffeine has been reported to affect normal calcium balance [Danielle, 1976; Heaney and Recker, 1982; Yeh and Aloia, 1986; Bergman et al., 1987]. Chronic administration of caffeine led to a negative calcium balance when there was an impaired ability to increase the efficiency of calcium absorption and led to a negative calcium absorption in elderly rats [Yeh and Aloia, 1986]. This is a situation which may be analogous to the effects of caffeine in the elderly human population, particularly in light of the findings of a slightly negative calcium balance in premenopausal women who have increased caffeine intake [Heaney and Recker, 1982]. Our studies, carried out in rats 4 weeks of age, revealed no changes in serum calcium and a suggested bone metabolism reflected by serum osteocalcin levels. How-

ever, in another report [Glajchen, 1988] an increase in serum osteocalcin was significant in a study with older rats, 11 weeks of age. Despite the changes in serum osteocalcin in older rats, a histomorphometric analysis of the bone showed no impairment in bone formation or bone resorption. Thus, from those studies and the data presented in this model it would appear that the effects of caffeine are targeted more to an impairment of developing fetal bone or development of the osteoblast phenotype and newly formed bone in the DBP implant model, and caffeine does not severely effect the normal turnover in the adult skeleton.

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