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BMP-6 Accelerates Both Chondrogenesis and Mineral Maturation in Differentiating Chick Limb-Bud Mesenchymal Cell Cultures

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Abstract Chick limb-bud mesenchymal cells, plated in micromass culture, differentiate in vitro to form a cartilaginous structure analogous to the epiphyseal growth plate. When inorganic phosphate, Pi, is included in the medium such that the total Pi concentration is 4 mM, apatite mineral precipitates around the ''hypertrophic'' chondrocytes. These hypertrophic chondrocytes are characterized by their increased expression of type X collagen, alkaline phosphatase activity, and apoptosis, as well as by the ability of their extracellular matrices to support mineral deposition. Under standard mineralizing conditions (0.8×10^6cells/micromass; 4 mM Pi, 1.3 mM Ca^{2+}, 10% FCS, and antibiotics) mineralization does not commence until day 14–16. Based on the ability of bone morphogenic protein 6 (BMP-6) to stimulate chondrocyte maturation in other systems, 100 ng/ml BMP-6 was added to chick limb-bud mesenchymal cell cultures 2 and 5 days after plating, and the effects of this addition on mineral accretion and the characteristics of the mineral and matrix determined. Addition of BMP-6 accelerated the differentiation of the mesenchymal cells to hypertrophic chondrocytes. In the presence of BMP-6 added on both days 2 and 5, mineralization (assessed on basis of ^{45}Ca uptake) commenced by day 12. Fourier transform infrared imaging (FTIRI) was used to monitor the mineral content and mineral crystallinity as a function of time from day 9 to 21 in cultures with and without exogenous BMP-6. While BMP-6 accelerated the rate of mineral accretion, and the crystals that were formed in the BMP-6 cultures were initially more mature, by day 21 the crystal size distribution in experimental and control cultures were not significantly different. This study, the first to report the detailed application of FTIRI to cell cultures, indicates the importance of the extracellular matrix in the control of crystal maturation. J. Cell. Biochem. 84: 509–519, 2002.

Key words: BMP-6; mineralization; crystal maturity; infrared imaging; mesenchymal cell culture; chondrocyte culture

Mesenchymal cells isolated from stage 21 to 24 chick limb-buds, and plated as a micro-mass (~1 million cells/20 μl spot) differentiate in culture to form a matrix resembling the growth plate in the chick [Binderman et al., 1979; Hadhazy and Szollosi, 1983; Solursh, 1989; Boskey et al., 1991, 1992a,b]. In the presence of 4 mM inorganic phosphate (Pi), 25 μg/ml ascorbic acid, 10% fetal calf serum (FCS), and antibiotics, the cells slowly hypertrophy, express type X collagen and alkaline phosphatase, and form a mineralized matrix [Boskey et al., 1992a,b]. In situ, the hypertrophic chondrocytes achieve a "terminal" maturation stage characterized by expression of increased levels of type X collagen [Linsenmayer et al., 1998, Lunstrum et al., 1999], vascular endothelial growth factor [Gerber et al., 1999; Horner et al., 1999], osteopontin [Lian et al., 1993; Barak-Shalom et al., 1995], osteonectin [Pacifici et al., 1990], matrix metalloproteinases (such as MMP-13) [D'Angelo et al., 2000], programmed cell death (apoptosis) [Gibson, 1998; Roach and Clarke, 1999; Anderson et al., 2000], and expression of bone morphogenic protein 6 (BMP-6) [Carey and Liu, 1995; Iwasaki et al., 1997; Grimsrud et al., 1999; Grimsrud et al., 2001; Kameda et al., 2000]. The hallmark of terminal differentiation is the deposition of a calcified extracellular matrix [Kato et al., 1988; Nie et al., 1998; Kirsch et al., 2000]. In the differentiating chick limb-bud mesenchymal cell system the onset

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of matrix mineralization occurs more than 11 days after plating [Boskey et al., 1991, 1992b, 1994, 1996a,b, 1997, 2000, 2001]. Based on the observation that mineral deposition occurred only around "hypertrophic" chondrocytes, it was hypothesized that mature, terminally differentiated chondrocytes, were required for mineral deposition, hence by accelerating hypertrophy, mineralization would be accelerated.

BMP-6 is a member of the 60A family of bone morphogenetic proteins (members of the TGF-beta superfamily) [Wharton et al., 1991]. The BMPs are known to induce differentiation of mesenchymal cells into chondroblast and osteoblast lineages, and to inhibit their differentiation into myocytes [Luyten et al., 1994; Kawabata et al., 1998; Fujii et al., 1999]. BMP-6 is expressed by hypertrophic chondrocytes [Carey and Liu, 1995; Imamura and Sakou, 1998; Terkeltaub et al., 1998; Yazaki et al., 1998; Matsunaga et al., 1999] as well as smooth muscle, nerve, and intestinal cells [Perr et al., 1999]. BMP-6 is involved in the developmental processes of renal and hepatic systems as well as of human fetal intestine [Perr et al., 1999]. BMP-6 induces osteoblastic differentiation of pluripotent mesenchymal stem cells and is an autocrine stimulator of chondrocyte differentiation [Wozney, 1992; Fujii et al., 1999; Grimsrud et al., 1999; Grimsrud et al., 2001; Aoki et al., 2001]. BMP-6 has been shown to accelerate maturation of cephalic sternal chondrocytes [Grimsrud et al., 1999]. The proliferating as well as the hypertrophic chondrocytes have BMP receptors [Zou et al., 1997; Enomoto-Iwamoto et al., 1998; Yazaki et al., 1998; Aoki et al., 2001]. Such functional receptors are required for maintenance of the hypertrophic phenotype as shown by dominant negative mutations in these receptors [Enomoto-Iwamoto et al., 1998]. BMP-6 activates alkaline phosphatase, acting through Smad1 and Smad5 and other signaling pathways discussed in detail elsewhere [Fujii et al., 1999; Aoki et al., 2001]. These results suggested that BMP-6 would autonomously maintain and/or promote a later stage of chondrocytic maturation [Kameda et al., 2000]. The goal of the present study was to use BMP-6 to increase the rate of maturation of the differentiating chick limb-bud mesenchymal cells into hypertrophic chondrocytes and compare the rate of mineral accretion and the properties of the mineral formed in the presence and absence of exogenous BMP-6.

MATERIALS AND METHODS

Culture System

Chick limb-bud mesenchymal cells were isolated from stage 21–24 [Hamburger and Hamilton, 1951] fertilized White Leghorn eggs (Truslow Farms, Chestertown, MD) as described in detail elsewhere [Boskey et al., 1992b]. Eggs were maintained in a humidified incubator at 37°C for 4.5 days. Embryos were then sterilely withdrawn from the eggs and their limb buds removed into 0.9% USP grade saline (Abbott Laboratories, N Chicago, IL). Cells were released from the limb buds by digestion with 5 ml 0.25 wt.% trypsin–0.53 mM EDTA (GIBCO, Grand Island, NY), and then were separated from debris by passage through two layers of 20 μm Nitex membrane (Tetko Inc., Ardsley, NY). Cells were counted with a hemocytometer, checked for viability by trypan blue dye exclusion, and pelleted in the cold at 2,300 rpm. In all cases, viability was greater than or equal to 98%. Cells were resuspended in medium containing 1.3 mM Ca and plated using the micro-mass technique [Ahrens et al., 1977] at a density of 0.8 million cells/20 μl drop in 35 mm × 10 mm Falcon dishes. Cells were allowed to attach for 2 h in a humidified atmosphere of 5% CO_2 at 37°C. After 2 h, Dulbecco's modified essential medium (DMEM; GIBCO Formula 80-0303A; Grand Island, NY) containing 1.3 mM calcium chloride, 1,000 mg/L glucose and supplemented with 50 units/ml penicillin and 25 μg/ml streptomycin, 10% fetal calf serum (GIBCO, Grand Island, NY), and 0.3 mg/ml glutamine was added. The day of plating was designated day 0. The medium was changed three times per week. Mineralizing cultures were supplemented with 3 mM inorganic phosphate from day 2 onward, making the total inorganic phosphate content 4 mM. From day 2 onward, and with every change of medium, 25 μg/ml sodium ascorbate (Sigma Chemicals, St Louis, MO) was added to the cultures. On days 2 and 5, experimental mineralizing and non-mineralizing cultures were treated with 1, 10, or 100 ng/ml BMP-6. Genetics Institute, Cambridge, MA, kindly provided the BMP-6. All experiments were repeated at least in triplicate, and each of the

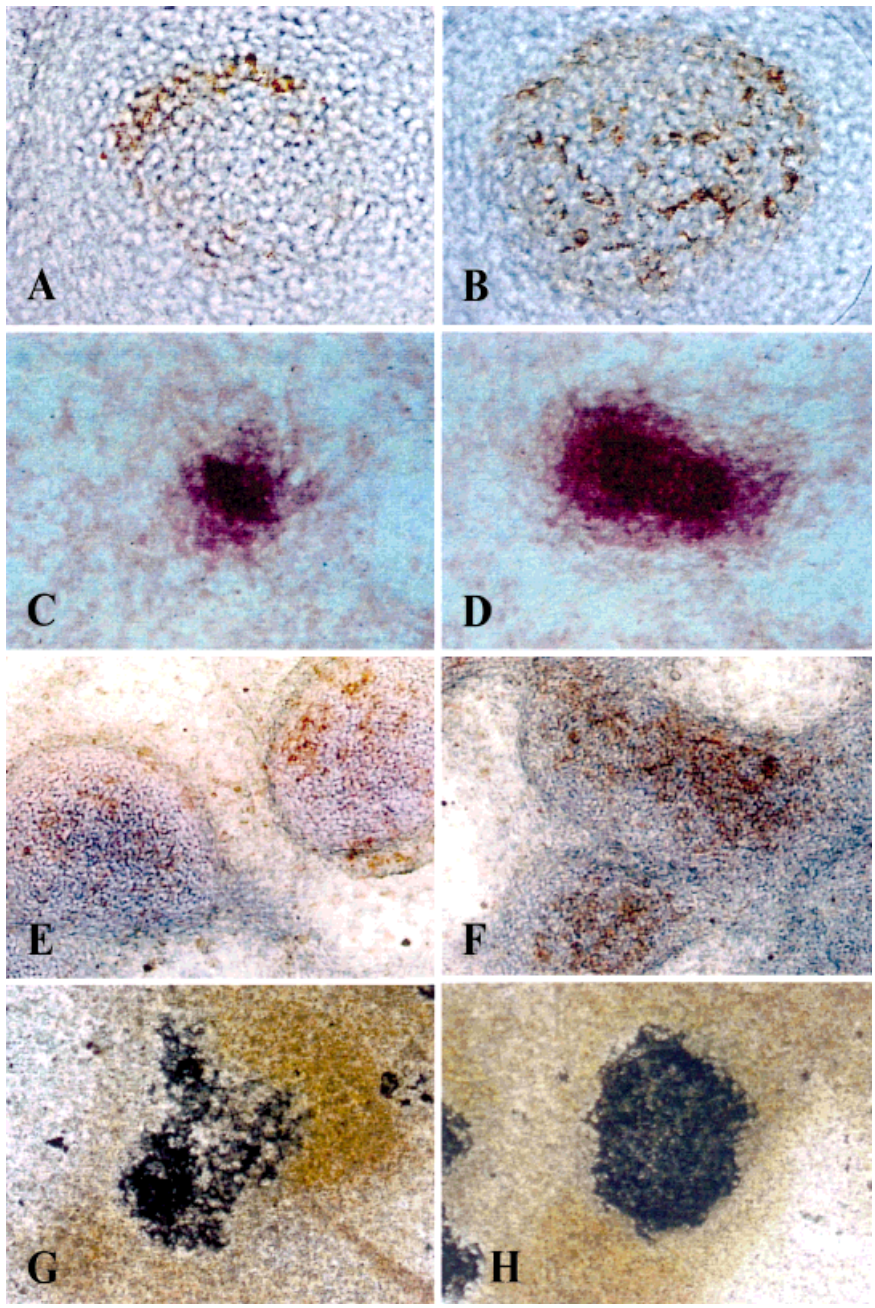

Fig. 1. Exogenous addition of BMP-6 (**B**,**D**,**F**,**H**) accelerates expression of phenotypic markers of hypertrophy compared with controls not supplemented with BMP-6 (**A**,**C**,**E**,**G**) at each time point examined. Typical data is shown for chondrocyte nodules in whole mount cultures for (A,B) type X collagen expression at day 12, (C,D) alkaline phosphatase activity at day 19, (E,F) apoptosis at day 16., and von Kossa reaction for deposited mineral (G,H) at day 19. In each case there is a qualitative increase in the BMP-6 treated cultures. Original magnification A, B = 48×; C-H = 120×.

triplicate experiments included three cultures at each time point.

Analytical Techniques

Light microscopy and immunohistochemistry were used to determine cell morphology, and localization of alkaline phosphatase activity and type X collagen protein. In brief, after removal of the medium, cultures were washed with sterile saline, then treated with 4% fresh para-formaldehyde in 0.05 M cacodylate buffer, pH 7.4, for 2 h. Cultures were then stored in cacodylate buffer plus 7% sucrose until analyzed. The analyses were performed on whole

cultures without sectioning on culture days 9–21. Alkaline phosphatase activity was demonstrated using the azo dye technique of Burstone [1961] staining at room temperature for 20–30 min. Type X collagen localization required a pretreatment of 1% hyaluronidase at 37°C for 45 min prior to immunostaining by the ABC method. The antibody (X-AC9, Linsenmayer) was obtained from the Hybridoma Bank, University of Iowa. Type I collagen localization utilized antibody from fetal mouse (Calbiochem; Cat # 234167) by the ABC method. Alcian blue staining (pH2) was used to demonstrate proteoglycan distribution. Additionally, the TUNEL assay was carried out using the TACS 2 TdT (Trevigen) method to estimate the distribution of apoptotic cells on days 9, 12, and 16 of culture.

The accumulation of mineral in the cultures was assayed, as detailed elsewhere [Boskey et al., 1992b, 1996a, 1997] based on ^{45}Ca uptake (expressed per total culture). In brief, ^{45}Ca uptake was determined for each micromass spot culture, following hydrolysis of the spot in 2N HCl (2 h, 60°C) and scintillation counting. The mineral accumulation was assessed on days 9–21. Data was expressed as the differential uptake per culture, where mean uptake in triplicate non-mineralizing cultures run at the same time under the same experimental conditions was subtracted from the mean uptake in triplicate similarly treated mineralizing cultures. Results were presented as mean and standard deviation from replicate experiments.

Mineral content and mineral maturity were mapped in non-mineralizing and mineralizing cultures on days 12–21 using FTIR imaging [Mendelsohn et al., 2000]. The cultures for these analyses were washed with saline, and air-dried on barium fluoride infrared windows (Spectra-Tech, Hopewell Junction, NY). Each culture was examined using the BioRad (Cambridge, MA) Sting-Ray^{TM} system. The instrument consists of a step-scan interferometer interfaced to an MCT focal plane array detector. There is a one-to-one optical mapping correspondence between each detector element and a 6.3 μm × 6.3 μm spot within the tissue which is located at the focal plane of an IR microscope. The sample size examined was 400 μm × 400 μm (64 elements × 6.3 μm per pixel in the x and y directions). A minimum of five images were mapped across the diameter of the culture. The 4,096 spectra per field were base-line corrected, and the ratios of the integrated areas of the phosphate band (~920–1,170 cm^{-1}) to the amide I band (~1,585–1,720 cm^{-1}) and the intensity ratio of subbands at 1,030 and 1,020 cm^{-1} calculated using a combination of BioRadWin IR-Pro, Origin Micro-calc, and Excel software. These ratios have been shown to correspond, respectively, to the ash weight (mineral content) [Pienkowski et al., 1997] and mineral maturity/crystallinity [Paschalis et al., 1997] in synthetic mixtures of apatites and collagen. Data were presented both as images of these parameters, and as histograms, representing the mean and standard deviations of the pixel distribution (percentage of total pixels per value) for multiple areas in multiple cultures. Collagen maturity was mapped from these spectra as the ratios of the intensity of the 1,660–1,690 cm^{-1} components [Paschalis et al., 2001].

Statistical Evaluation

Mean and standard deviations were calculated, and significant differences between experimental and control conditions evaluated based on ANOVA and the appropriate statistical test. A $P \leq 0.05$ was taken as significant.

RESULTS

The cultures treated on day 2 or 5, or days 2 and 5, with 1 and 10 ng/ml BMP-6 showed no significant differences in morphology or ^{45}Ca uptake (data not shown). Cultures treated with

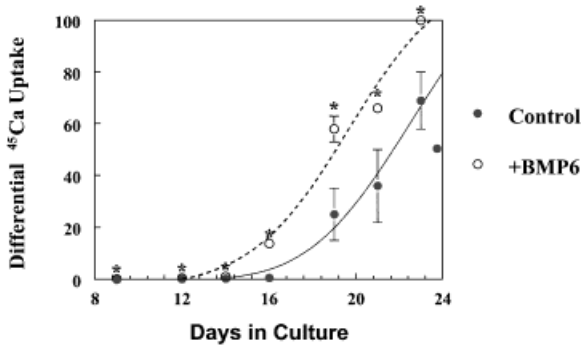

Fig. 2. Differential ^{45}Ca uptake, calculated as the difference between ^{45}Ca uptake in similarly treated mineralizing and non-mineralizing cultures. Data was normalized to the day 23 values in the BMP-6 treated cultures, and is presented as mean ± SD for 3–5 independent cultures. *$P \leq 0.05$, compared to cultures at same time point given exogenous BMP-6.

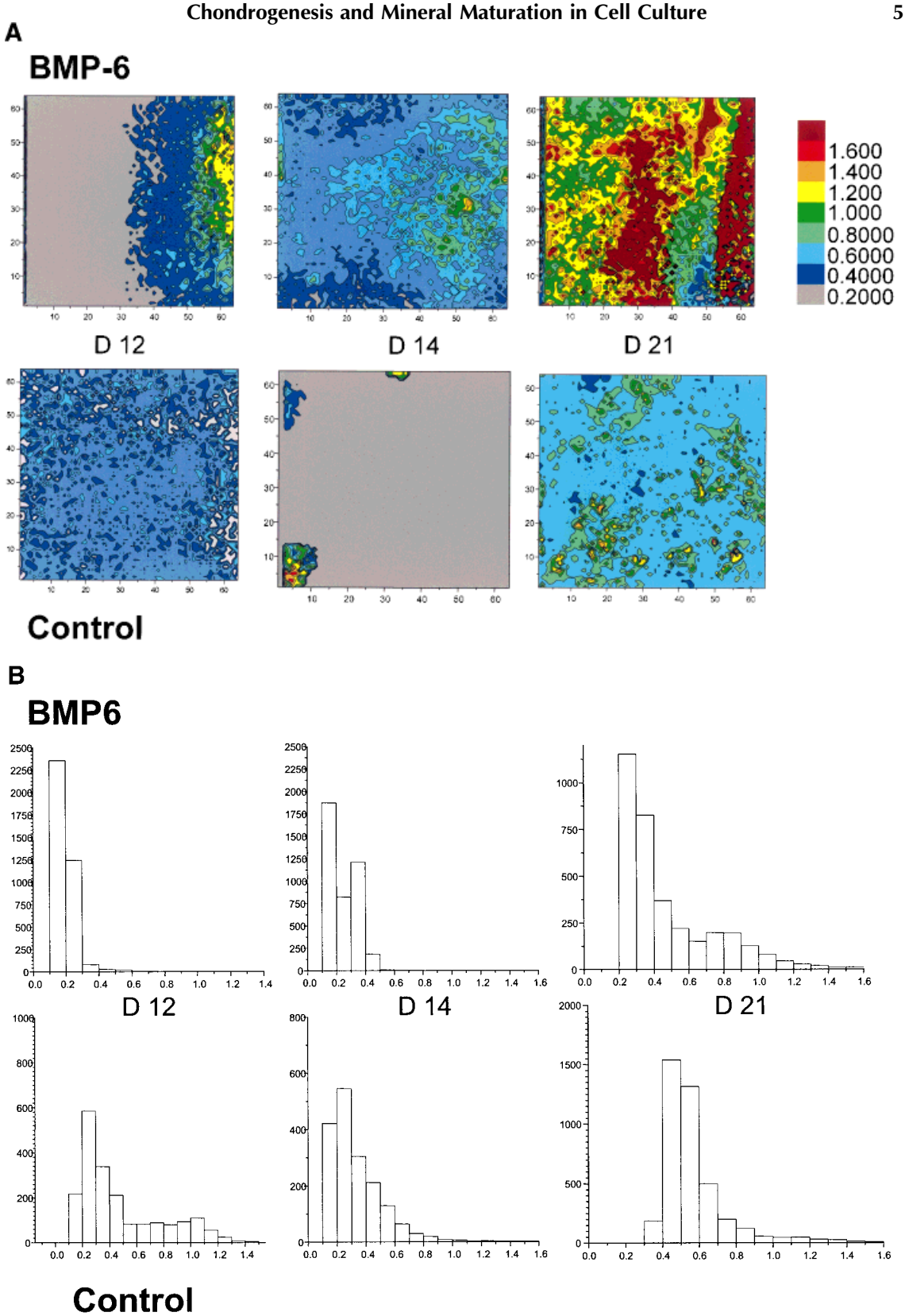

Fig. 3. **A**: Typical infrared images of the mineral to matrix ratio at a site on the edge of the culture as a function of time, in mineralizing BMP-6 treated (top) and control (bottom) cultures. The image shows the data for 64×64 pixels in the array detector. All images are presented with the same color scale (right hand bar). **B**: Histograms for these individual images indicate the number of pixels in each individual image that have the indicated mineral to matrix value. **C**: The mean $\pm$ SD for the time dependent changes in mineral to matrix ratio at all sites in all control (no exogenous BMP-6) or BMP-6 treated at each time point. The significant increase in the BMP-6 treated cultures is noted.

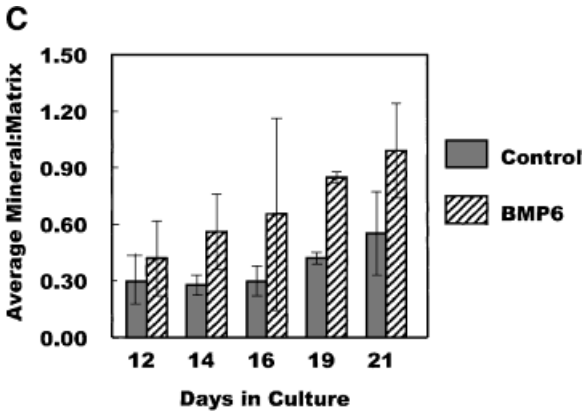

Fig. 3. *(Continued)*

100 ng/ml on both days 2 and 5 showed significant differences, indicating increases in the extent of maturation and mineralization, hence those results will be presented here. Figure 1 which shows chondrocyte nodules in whole mount cultures compares typical markers of chondrocyte hypertrophy at representative time points. The BMP-6 treated cultures were consistently more mature. For example, addition of exogenous BMP-6 increased type X collagen expression (Fig. 1B), and alkaline phosphatase activity (Fig. 1D) compared to non-supplemented cultures (Fig. 1A and D). The BMP-6 treated cultures showed more positive TUNEL staining implying there was an increased distribution of apoptotic cells (Fig. 1F) in those cultures which received exogenous BMP-6 than those that did not (Fig. 1E).

Addition of exogenous BMP-6 also changed the distribution of other matrix constituents. There was increased staining for type I collagen in BMP-6 treated cultures as contrasted with cultures not receiving exogenous BMP-6 (not shown). Qualitative mineral distribution was also different, with more positive von Kossa staining in the BMP-6 treated cultures (Fig. 1H) contrasted with cultures without added BMP-6 (Fig. 1G).

Based on quantitative measurements, there was a significant acceleration of the ^{45}Ca uptake in the presence of exogenously treated cultures, as shown by the differential uptake in Figure 2 Extrapolation of the linear portion of the curve revealed that initial mineralization in the BMP-6 treated cultures commenced at almost day 12–13 in contrast to almost day 16–17 in the cultures not receiving BMP-6.

Infrared imaging analyses (Fig. 3) of the mineral content of the cultures as a function of time confirmed the ^{45}Ca uptake data. Typical images of the mineral to matrix ratio at the edge of the cultures treated with exogenous BMP-6 and untreated mineralizing cultures are shown for days 12, 14, and 21 in Figure 3A. Histograms showing the shift in mineral to matrix ratio distribution as a function of culture age are shown in Figure 3B. Figure 3C compares the mean mineral to matrix ratio in the entire culture in the presence and absence of exogenous BMP-6 as a function of time.

Analysis of the mineral maturity (crystallinity), based on the intensity ratio of bands at 1,030 and 1,020 cm^{-1} demonstrated that BMP-6 accelerated the rate at which crystals matured. However, the mean crystallinity of the mineral formed at the completion of the study was comparable to that of the cultures to which exogenous BMP-6 was not added. The images of spatial distribution of crystallinity in Figure 4A demonstrate that while at day 14 the BMP-6 treated cultures showed markedly increased crystallinity, by day 21 the distributions are similar. This is confirmed in the histograms in Figure 4B, and the mean crystallinity from all images analyzed in Figure 4C.

Maturity of the collagenous matrix, both throughout the center of cultures (Fig. 5A) and on the periphery of the culture (Fig. 5B) showed a significant linear increase with time ($P \leq 0.01$). However while there was a more uniform distribution of this parameter in BMP-6 treated cultures, there were no significant differences in collagen maturity in BMP-6 treated and untreated cultures at any given time point.

DISCUSSION

This study has shown that exogenous BMP-6 addition not only accelerates maturation of chondrocytes, but also, in support of our hypothesis, demonstrates that this increase in cell maturation is associated with earlier initiation of mineralization. Of greatest interest was the finding that the final mineral formed in the presence of BMP-6 was not more mature than that formed in the absence of exogenously added BMP-6. Data support the hypothesis that mature chondrocytes are required for mineralization and, more importantly suggest that BMP-6 can be used to stimulate chondrocyte-mediated mineralization.

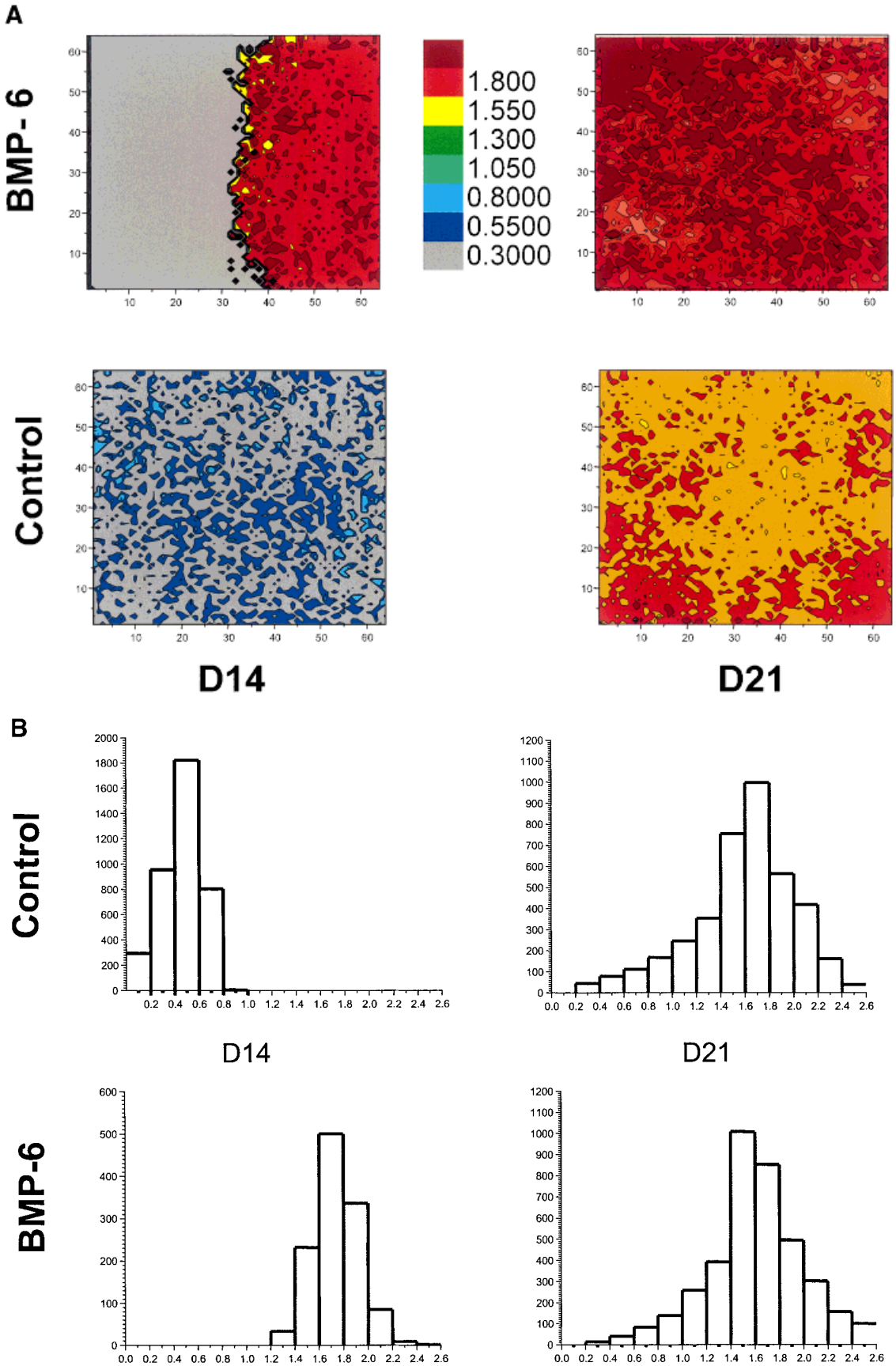

Fig. 4. **A**: Infrared images demonstration the crystallinity (1,030:1,020 cm^{-1} intensity ratio) in BMP-6 supplemented (top) and control (without added BMP-6) cultures (bottom) at days 14 and 21. All images are presented with the same color scale. **B**: Histograms for these individual images indicate the number of pixels in each individual image that have the indicated crystallinity. **C**: The mean crystallinity as a function of culture age in all cultures examined. There is no significant difference between the crystallinity of the cultures by day 21.

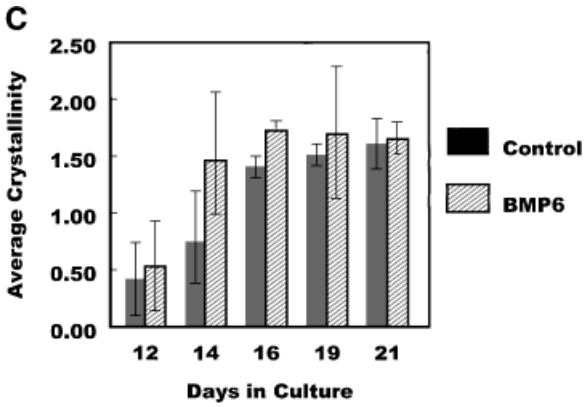

Fig. 4. *(Continued)*

Other investigators had demonstrated that BMP-6 addition stimulated chondrocyte hypertrophy [Grimsrud et al., 1999; Grinsrud et al., 2001], albeit not in the differentiating chick limb-bud culture system. However, high density differentiating mesenchymal cell cultures do mature more rapidly when treated with other BMPs [Carrington et al., 1991]. The finding in the present study that type X collagen and alkaline phosphatase expression was accelerated by exogenous BMP-6 addition is in agreement with earlier reports in the literature [Grimsrud et al., 1999; Gitelman et al., 1994]. An increase in apoptosis had also been reported to be associated with the BMP stimulation of chondrocyte hypertrophy [Buckland et al., 1998].

During endochondral ossification, mineral appears around the bottom-most hypertrophic chondrocytes. It is not clear, however, whether the matrix produced by the hypertrophic chondrocytes facilitates this mineralization, or whether the vascular invasion below the so-called zone of provisional ossification results in an increase in the local CaxP concentration facilitating mineralization, or both. In the differentiating mesenchymal cell culture system, where there is no vascular invasion, mineralization can occur when the CaxP product exceeds 5 mM^2 [Boskey et al., 1992b]. Modification of the matrix (removal of proteoglycans [Boskey et al., 1997], decreased or increased proteoglycan sulfation [Boskey et al., 1997, 2001], inhibition of protein kinases [Boskey et al., 1994], and immuno-blocking type I collagen [Boskey et al., 2000]) alters the time at which mineralization commences and the properties of the mineral. It is thus likely that an appropriate matrix must be present for mineralization to commence. To make such a matrix, cells of appropriate maturity must also be present. By accelerating the progression of the cells to the hypertrophic, terminally differentiated state, mineralization was facilitated. Additional studies in which immature cells are plated on a mature matrix, and vice versa, will be required to fully validate that hypothesis.

The mineral crystals found in calcified cartilage [Arsenault and Grynpas, 1988] as well as those in bone [Bonar et al., 1983; Handschin and Stern, 1994] undergo significant changes in crystallite size and perfection during development, but change relatively slightly after bone growth ceases. The average size of these crystals is relatively invariant. This implies that the regulation of crystal formation and growth is not only a physicochemical process, but is controlled by matrix proteins [Boskey, 2001].

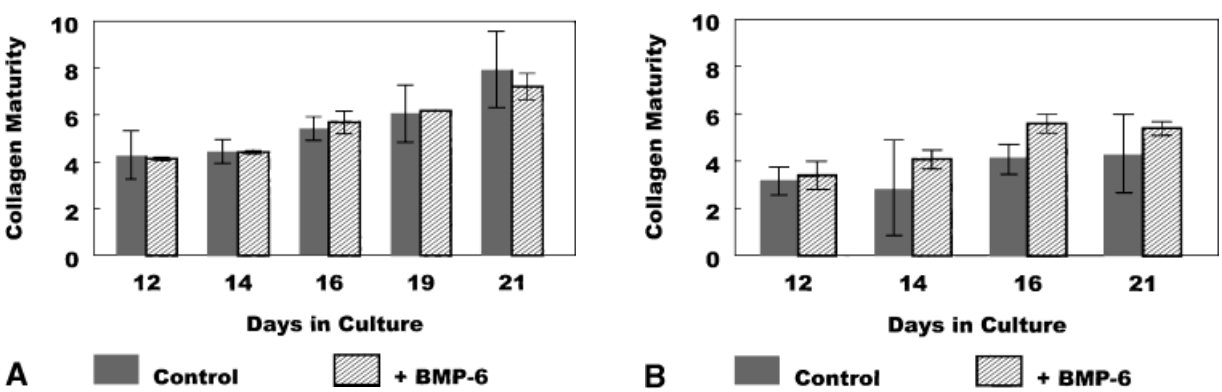

Fig. 5. **A**: Collagen maturity (1,660:1,690 cm^{-1} ratio) in the centers of replicate cultures increases with time in cultures with and without exogenous BMP-6, values are mean + SD, N^{3-} cultures, four images per culture. **B**: Collagen maturity at periphery of micro-mass nodules increases with time in cultures with and without exogenous BMP-6.

Were mineral crystal maturation dependent only on local ion concentrations, in those cultures in which Ca and P concentrations are being maintained with every change of medium, one would expect to see both proliferation and growth of crystals. In contrast, in the cultures studied here, the crystals seemed to achieve their "optimal" size, and not grow beyond that. We have also examined mineral crystals in cultures grown for 23 –27 days [unpublished], and there does not appear to be an increase in mineral crystal size beyond that seen on day 21, although the smaller crystals continue to mature, making the distribution of crystal size and perfection less broad. In mineralized cultures in which the cells were lysed releasing both degradative enzymes and additional calcium and phosphate ions in to the matrix [Boskey et al., 1996a], or in mineralizing cultures in which proteoglycans were degraded [Boskey et al., 1997], crystal size (as determined by X-ray diffraction and infrared microspectroscopy) was larger than that in untreated cultures. Thus it is apparent that the matrix is responsible for modification of the crystal size. The panoply of specific proteins responsible for regulation of these sizes is still under investigation.

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