Journal of Cellular Biochemistry 79:89–102 (2000)

Type I Collagen Influences Cartilage Calcification: An Immunoblocking Study in Differentiating Chick Limb-bud Mesenchymal Cell Cultures

A.L. Boskey,* D. Stiner, I. Binderman, and S.B. Doty

The Hospital for Special Surgery, New York, New York 10021

Abstract Chick limb-bud mesenchymal cells, plated in high-density micro-mass culture, differentiate and form a matrix resembling chick epiphyseal cartilage. In the presence of 4 mM inorganic phosphate or 2.5 mM β-glycerophosphate mineral deposits upon this matrix forming a mineralized tissue that, based on electron microscopy, x-ray diffraction and Fourier Transform Infrared microspectoscopy, is like that of chick calcified cartilage. In this culture system the initial mineral deposits are found on the periphery of the chondrocyte nodules. During differentiation of the cells in the high-density micro-mass cultures there is a switch from expression of type I collagen to type II, and then to type X collagen. However, type I collagen persists in the matrix. Because there is some debate about whether type I collagen influences cartilage calcification, an immunoblocking technique was used to determine the importance of type I collagen on the mineralization process in this system. Studies using nonspecific goat anti-chick IgG demonstrated that 1–100 ng/ml antibody added with the media after the cartilage nodules had developed (day 7) had no effect on the accumulation of mineral in the cultures. Nonspecific antibody added before day 7 blocked development of the cultures. Parallel solution based cell-free studies showed that IgG did not have a strong affinity for apatite crystals, and had no significant effect on apatite crystal growth. Type I collagen antibodies (1–200 ng/ml) added to cultures one time on day 9 (before mineralization started), or on day 11 (at the start of mineralization), slightly inhibited the accumulation of mineral. There was a statistically significant decrease in mineral accretion with 100 or 200 ng/ml collagen antibody addition continuously after these times. Fab' fragments of nonspecific and type I collagen antibodies had effects parallel to those of the intact antibodies, indicating that the decreased mineralization was not attributable to the presence of the larger, bulkier antibodies. The altered accumulation of mineral was not associated with cell death in the presence of antibody (demonstrated by fluorescent labeling of DNA) or with increased apoptosis (TUNEL-stain). In the immunoblocked cultures, EM analysis demonstrated that mineral continued to deposit on collagen fibrils, but there appeared to be fewer deposits. The data demonstrate that type I collagen is important for the mineralization of these cultures. J. Cell. Biochem. 79:89–102, 2000.

Key words: type I collagen; mineralization; chondrocyte culture; immunoblocking; matrix vesicle

Chick limb-bud mesenchymal cells, plated in micro-mass culture, differentiate and form a matrix that resembles chick epiphyseal cartilage [Binderman et al., 1979; Hadhazy et al., 1982; Boskey et al., 1992]. In the presence of 4 mM inorganic phosphate or 2.5 mM β-glycerophosphate (βGP), mineral deposits on this matrix, forming a mineralized tissue that, based on x-ray diffraction electron microscopy (EM) and Fourier Transform Infrared microspectoscopy is like that of chick calcified cartilage [Boskey et al., 1992a, 1996]. Thus, this culture system and analogous ones using rodent mesenchyme have been used

Grant sponsor: NIH; Grant number: AR037661.

Dr. Boskey designed the study, obtained the funding for the study, performed the mineral analyses, interpreted all results, and wrote and edited the paper.

Dr. Doty performed the immunohistochemical studies and revised and edited the manuscript.

Dr. Binderman assisted in the interpretation of results and revised and edited the manuscript.

Ms. Stiner performed all the cell culture experiments, did the isotope based studies, and assisted in the preparation of sections for histology. She also edited the manuscript.

*Correspondence to: Adele L. Boskey, The Hospital for Special Surgery, 535 East 70th Street, New York, NY 10021. E-mail: Boskeya@hss.edu

Received 28 October 1999; Accepted 5 April 2000

extensively to model both cartilage differentiation and endochondral ossification [Daniels et al., 1996]. In this culture system, the initial mineral deposits are found on the periphery of the chondrocyte nodules [Boskey et al., 1992b]. Electron microscopy demonstrated that the peripheral mineral is associated with thicker collagen fibrils [Boskey et al., 1992a], shown by immunohistochemistry to be type I collagen [Schroter-Kerman et al., 1991]. The mineral closer to the nodules is associated with thinner collagen fibrils, presumably type II collagen [Kosher et al., 1986; Langille et al., 1990], and with extracellular matrix vesicles [Boskey et al., 1992a]. In similar differentiating mouse limb bud mesenchymal cell micro-mass cultures, a similar pattern of types I and II localization is noted [Edvall-Arvidsson and Wroblewski, 1996].

The importance of type I collagen in the initial calcification of the growth plate has been extensively debated [von der Mark and von der Mark, 1977; Yasui et al., 1984; Bianco et al., 1998; Nie et al., 1999]. Reports that mineral is associated with both type II and type I collagen seem to be both species and site specific. The human growth plate hypertrophic chondrocytes lack type I collagen expression [Kirsch and von der Mark, 1992; Reichenberger et al., 1991], while in the mouse and the avian systems type I collagen expression by hypertrophic chondrocytes has been reported [Ishii et al., 1998; Kosher et al., 1986]. Even in the avian system, condylar cartilage hypertrophic cells do not express type I collagen while epiphyseal hypertrophic cells do [Ishii et al., 1998]. The other debated issue concerns the relative importance of collagen (and its associated matrix proteins) and extracellular matrix vesicles in the initiation of cartilage calcification [Anderson, 1995; Glimcher, 1989]. The goal of the present investigation was to test the hypothesis that type I collagen was involved in mineralization of the matrix produced by differentiating chick limb-bud mesenchymal cells in culture. Concurrently, we sought to validate the technique of immunoblocking for studies of in vitro mineralization.

MATERIALS AND METHODS

Cell Culture Studies

Chick limb-bud mesenchymal cells were isolated from stage 21–24 [Hamburger and Hamilton, 1951] fertilized White Leg Horn eggs (Truslow Farms, Chestertown, MD) as described in detail elsewhere [Boskey et al., 1992a]. The eggs were maintained in a humidified incubator at 37° for 4.5 days. The 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, released from the limb buds by digestion with 5 ml 0.25 wt % trypsin - 0.53 mM EDTA (GIBCO, Grand Island, NY) 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 ≥ 98%. Cells were resuspended in medium containing 1.3 mM Ca and plated using the micro-mass technique [Ahrens, 1977] at a density of 0.75 million cells per 20 μl drop in 35 × 10 mm Falcon dishes and allowed to attach for 2 h in a humidified atmosphere of 5% CO_2 at 37°. 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), 25 μg/ml ascorbic acid, and 0.3 mg/ml glutamine was added. Mineralizing cultures were supplemented with either 3 mM inorganic phosphate or 2.5 mM β-glycerophosphate from day 2 onward. The rate of mineral accretion with these two additives and the pattern of mineralization are comparable, although phosphate does not start to accumulate in the β-glycerophosphate treated cultures until day 9 [Boskey et al., 1996]. Control cultures received no phosphate supplements. Media was changed every 48 h. Cultures were maintained for 21–23 days with day 0 referring to the day of plating.

Antibody Addition

Nonspecific goat antichick IgG was obtained from Sigma Chemicals (St. Louis,

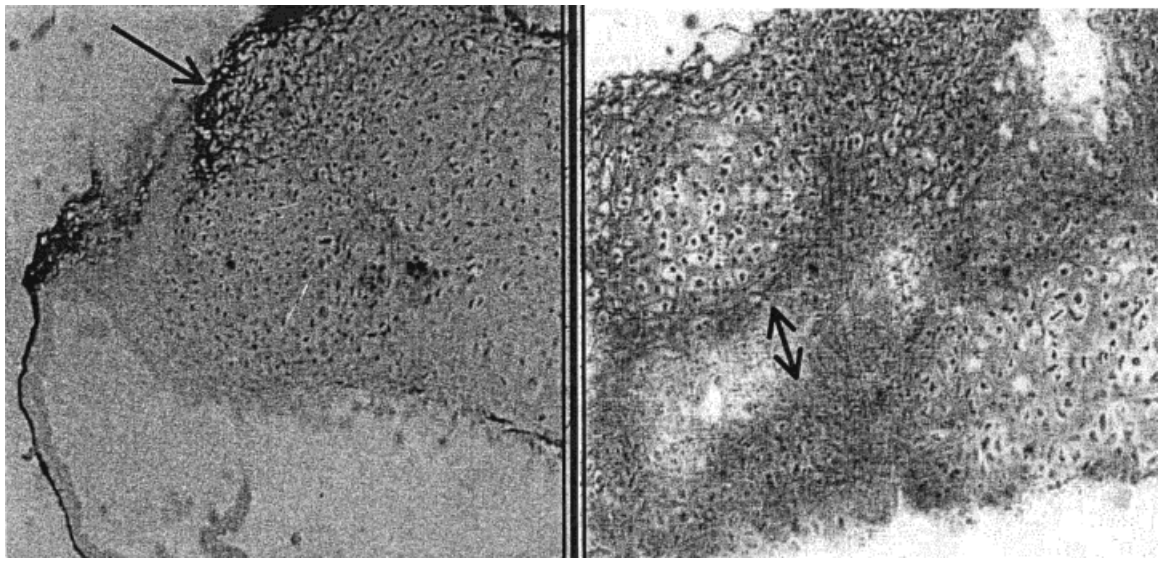

Fig. 1. Distribution of type I collagen in the mineralizing cultures at day 16. The type I collagen antibody (localized with biotin labeled anti-IgG, colocalized with streptavidin conjugated to horseradish peroxidase, and visualized with the diaminobenzidine) is localized around the periphery of the nodules. Left panel is cross-section, right panel is longitudinal section. Arrows point to periphery. Tissues counterstained with methyl green.

MO). Anti-chick type I collagen polyclonal antibodies and antichick type III collagen polyclonal antibodies were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). The goat anti-type I collagen is reported to show less than 10% cross-reactivity with types II, III, IV, V and VI collagen (Southern Biotechnology Associates, Inc.). Fab' fragments of each of these antibodies were prepared by pepsin digestion as described by Brackenbury et al. [1977]. In brief, the antibody was dissolved (10 mg/ml) in 0.1M sodium acetate and incubated with 5mg/ml twice crystallized pepsin (Worthington Biochemical Corp., Lakewood, NJ) for 24 h at 37°. The product was converted to monovalent Fab' by successive repeated dialyses against 0.1M 2-mercaptoethanol in phosphate buffered saline (PBS), then against 0.1M iodoacetamide in PBS, and then against PBS. One ml aliquots containing ≈800 ng/ml Fab' were stored at -20° until use. The effectiveness of the pepsin cleavage was documented by SDS-PAGE [Gallagher and Smith, 1995] with Coomassie and silver staining, and Fab' concentrations confirmed by the Bradford dye binding assay [BioRad, Cambridge, MA]. For these analyses, the intact antibodies (400 μg/ml) and the Fab' fragments (≈200 μg/ml) were loaded as 5 and 30 or 10 and 60 λ aliquots.

Specific and nonspecific antibodies were added to the cultures in concentrations from 1–200 ng/ml either acutely, on a single day, or continuously with each media change. Addition commenced on day 5, 7, 9, 11, or 16 of culture.

Immunocytochemistry

The cultures at day 16 or 21 were preserved in 70% ethyl alcohol or aldehyde fixative (2% paraformaldehyde plus 0.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4) for 2 h at room temperature. Tissues were rinsed in phosphate buffered saline (PBS, pH 7.4), blocked with DAKO serum-free blocking agent, and treated with goat anticollagen type I (Southern Biotechnology, Inc.). The primary antibody was used at 1:10 dilution in PBS for 18 h at 4°C. Tissues were rinsed in PBS, treated with biotin labeled anti-IgG for 1 h at room temperature, rinsed in PBS, and then treated with streptavidin conjugated to horseradish peroxidase for 1 h at room temperature. Antibody localization was visualized with the diaminobenzidine reaction in PBS for 10 min. Tissues were counterstained with methyl

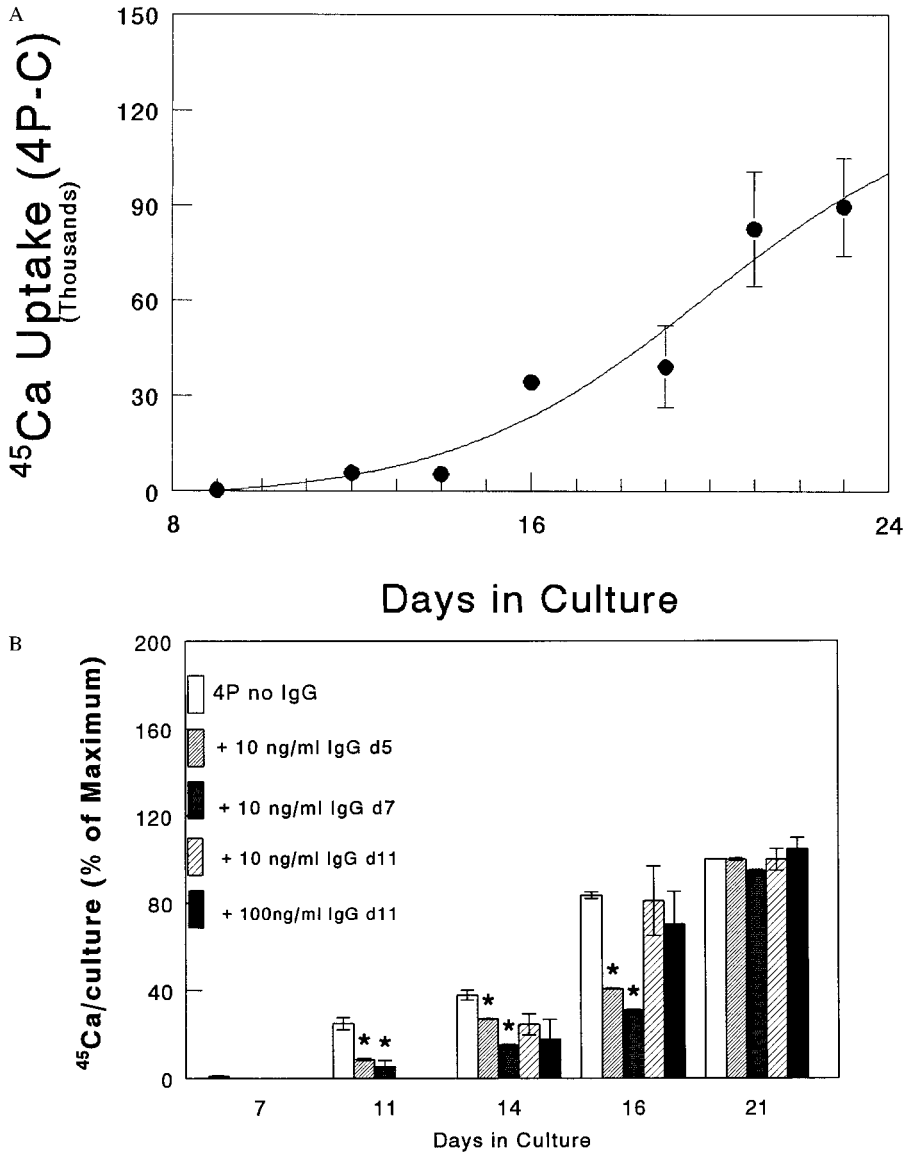

Fig. 2. Nonspecific Antibody Effects on Calcium Accumulation. **A:** Differential ^{45}Ca uptake (mineralizing(4P) - control(C)) in the mineralizing culture systems for all antibody-free experiments (n = 24), demonstrating the reproducibility, and justifying the normalization to maximum uptake used in other figures. **B:** Nonspecific IgG added after day 7 did not affect calcium uptake. Symbols indicate date of addition, and concentration (ng/ml) added. **C:** Addition of Fab' fragments of nonspecific IgG on day 9 or day 12 does not effect calcium uptake. **D:** Type III collagen antibodies added from day 9 in concentrations of 0, 10, 100, and 200 ng/ml culture medium did not alter Ca accumulation. Values for Figure 2B–D include both total calcium and ^{45}Ca uptake, and have been normalized to express percent of maximum uptake at day 21. All data indicate the differential uptake (mineralizing minus control) at each time point. Mean ± SD for a minimum of three different experiments is presented. *$P \leq 0.05$ relative to antibody free control at same time point.

green. Specificity of the type I antibody was confirmed by its reaction with rat tendon and chick cortical bone.

Cell Viability and Apoptosis

To determine whether the antibodies were altering cell viability, cultures were collected at day 16 or 21 and stained for cell viability and the distribution of apoptotic cells. Cell viability was determined with the Live/Dead viability kit available from Molecular Probes (Eugene, OR). In this method, a cell permeant substrate enters live cells and is

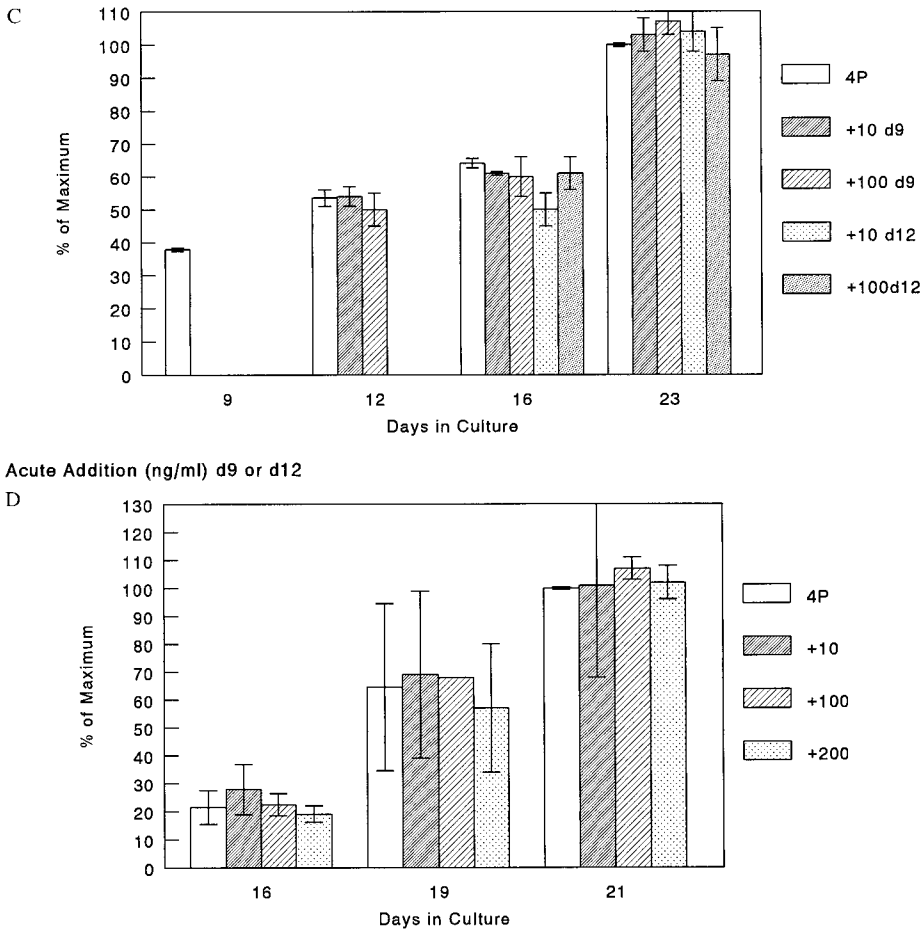

Figure 2. (Continued.)

cleaved by intracellular esterase activity to produce a green fluorescent signal. Nuclei of all cells, dead or alive, are stained with ethidium bromide, which causes all the nuclei to fluoresce red. The cultures are rinsed with PBS and stained for 1–5 min in these two reagents, then photographed with fluorescent microscopy using appropriate filters for the green cytoplasmic stain and the red nuclear stain. To determine the number of cartilage cells undergoing apoptosis, the end labeling of DNA fragments was examined using the Apoptag Kit (Oncor, Gaithersberg, MD). The staining method was applied to frozen sections of the alcohol or aldehyde fixed cultures [Hatori, 1995].

Mineral Analyses

The accumulation of mineral in the cultures was assayed, as detailed elsewhere [Boskey et al., 1992a; Boskey et al., 1997], based on ^{45}Ca uptake (expressed per DNA or per total culture), or total calcium uptake, determined by atomic absorption spectrophotometry [Willis, 1960]. Mineral uptake was calculated after correcting for calcium accumulation in nonmineralizing, similarly treated cultures, sampled at the same time point. This correction is necessary because in this culture system the cartilage matrix itself binds calcium [Boskey et al., 1991]. To compare results based on the two different calcium assays, most data is presented as a fraction of the maximum in mineralizing cultures (day 21 or 23) that did not receive antibodies. In a limited number of cultures, the presence of hydroxyapatite mineral was confirmed at day 21 by electron microscopy [Boskey et al., 1992a], FTIR microspectroscopy

[Boskey et al., 1992b], and x-ray diffraction [Boskey et al., 1992a]. For the FTIR studies, the cultures collected at day 21 were cryosectioned directly on to barium fluoride windows, and spectra recorded at 20 μm resolution using a BioRad Infrared Spectrometer (Cambridge, MA) with an MCT detector.

Statistical Evaluation

Each experiment was done at least in triplicate, with multiple (3–5) dishes of each condition and each time point for each experiment. Mean and SD 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.

Cell-Free Apatite Growth Studies

The rate of calcium and phosphate uptake in the presence of 0–20 mg/ml nonspecific human IgG (Sigma Chemicals, St. Louis, MO) and chick type I collagen antibodies was monitored at 37°C. Apatite "seed" crystals (0.5 mg/ml) of known specific surface ($54 m^2/gm$) were incubated in a metastable calcium phosphate solution containing 1.3 mM calcium chloride, 0.4 mM magnesium chloride, ≈1.7 mM $Na_2H_2PO_4$, 0.25 mM NaH_2PO_4, 117 mM NaCl, and 0.05 mM Tris buffer (pH 7.4), and 1.0 mM ammonium acid phosphate in 0.15M Tris-buffer containing the antibody. Aliquots were removed at time 0 (before the addition of seeds) and every 15 min for the next hour, hourly for the next 6 h, and at 24 h. Following centrifugation (2,300g) in a Beckman microfuge, samples were collected for measurement of total protein (BioRad Dye Binding Assay, Pierce Chemicals, Brooklyn NY), calcium [Willis, 1960], and inorganic phosphate [Heinonen and Lahti, 1981]. For the nonspecific IgG studies, solutions contained 0–18 mg/ml human IgG. Since the IgG preparations (supplied in phosphate-buffered saline) increased the phosphate concentration of the test solutions, each experiment had its own control, with adjusted to that of the IgG containing solution by preliminary analysis of phosphate contents [Heinonen and Lahti, 1981]. The type I collagen antibody was dissolved in Tris buffer, therefore phosphate modification was not needed. Each experiment was repeated in triplicate, and values expressed as mean ± SD for n = 3 independent experiments.

The binding affinity for apatite was calculated using the Langmuir adsorption isotherm model for data obtained at 37° as described elsewhere [Hay and Moreno, 1979; Boskey and Dick, 1991]. In brief, 0.5–10 mg/ml apatite crystals were incubated with 0–100 μg/ml IgG for 24 h at 37°. The 24-h period was selected following pilot studies which indicated binding equilibrium was achieved in ≈16 h. The IgG concentration of the initial solution (Co) and the equilibrium concentration after adsorption to the apatite (Ce) were measured using the Bradford dye binding protein assay (BioRad), with IgG as standard. The amount of IgG bound per surface area (Q) was calculated using the specific surface of the apatite ($54 m^2/gm$). An isotherm was plotted, and the linearized form of the Langmuir adsorption isotherm constructed as described elsewhere [Hay and Moreno, 1979]. The linearized form was written:

$$Ce/Q = 1/NK + Ce/N$$

in which N is the maximum number of adsorption sites per unit surface area of the adsorbent, and K is an estimate of the affinity of the absorbate for those sites.

RESULTS

Figure 1 shows the distribution of type I collagen in the mineralizing differentiating mesenchymal cell micro-mass cultures at day 16. As can be seen from the dark staining of the second antibody, type I collagen is found at the periphery of the chondrocyte nodules. Earlier studies demonstrated that the type I collagen gene is expressed throughout the culture period [Boskey et al., 1992a; Kosher et al., 1986].

In the mineralizing cultures, in the absence of added antibody, differential calcium uptake (calculated as the difference between ^{45}Ca accumulation in mineralizing and identically treated control cultures) is extremely reproducible. Figure 2A shows the precision of these results from 24 independent experiments. The shape of the curve based on total calcium accumulation was indistinguishable from this curve (data not shown). To compare calcium uptake in the presence and absence of additives, differential calcium uptakes are ex-

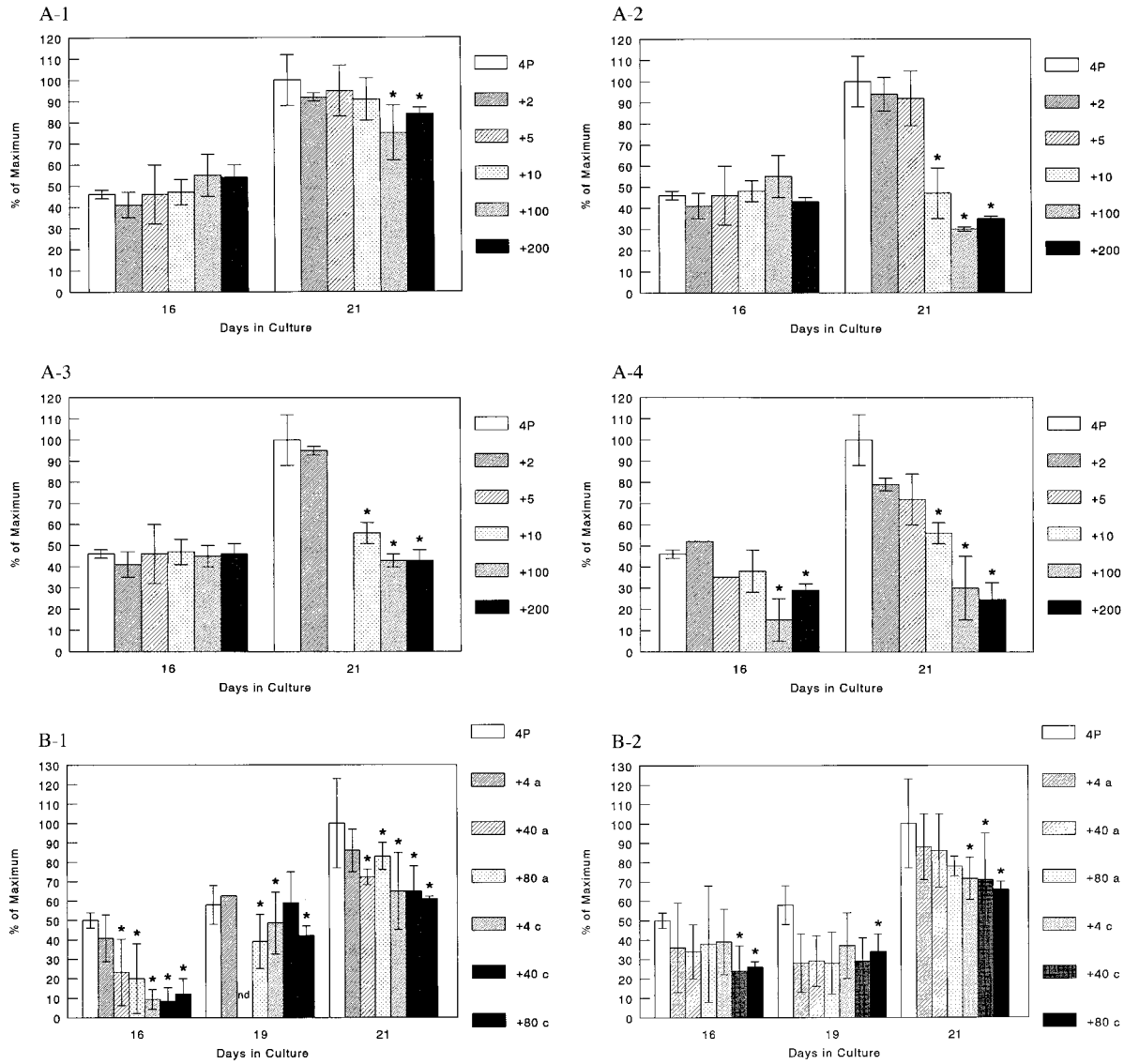

Fig. 3. Addition of Type I Collagen Antibodies Blocks Mineral Accumulation. **A:** Intact antibody addition acutely on day 9 (A-1) or continuously on day 9 (A-2), acutely on day 11 (A-3), or continuously on day 11 (A-4) decreases calcium uptake. **B:** Fab' fragment addition acutely (A), or continuously (C) on day 9 (B-1), or day 11 (B-2) similarly blocks calcium uptake. All values are Mean ± SD. Values include both total calcium and ^{45}Ca uptake, and have been normalized to express percent of maximum uptake at day 21. All data indicate the differential uptake (mineralizing minus control) at each time point. Mean ±SD for a minimum of three different experiments is presented. * indicates significant difference from cultures without antibodies at the same time point, $P \leq 0.05$, Bonferroni multiple comparisons test.

pressed as percentage of the day 23 or day 21 maximum in mineralizing cultures that did not receive antibodies. As seen in Figure 2B and C, adding a nonspecific goat anti-chick IgG to the cultures at different time points both as the intact antibody (Fig. 2B) and its Fab' fragments (Fig. 2C) had no effect on calcium accumulation when the antibody was added after day 7. Earlier addition markedly decreased mineral accumulation (not shown) and retarded the growth of the cultures. Similar effects were seen whether addition was acute or chronic. Comparably, addition of an antibody to a collagen that is not mineralized (type III collagen) also had no effect on calcium accumulation (Fig. 2D).

In contrast, when the type I collagen antibodies were added, either acutely or continuously, after day 9 or 11, there was a decrease in the amount of mineral accumulated (Fig. 3A).

As seen, the inhibition was greatest for continuous addition of 200 ng/ml antibody, while there was a slight but not consistently significant decrease with 10 ng/ml. The Fab' fragments stock concentration was 160μg/ml, thus as indicated in Figure 3B, 4, 40, or 80 ng/ml was added to the cultures. As with the higher concentrations of the intact antibody, continuous addition of Fab' fragments was more effective in blocking calcium uptake than a single addition, either at day 9 (Fig. 3B-1) or day 11 (Fig. 3B-2). Whether intact antibody was added, or Fab' fragments the decrease in calcium uptake with antibody addition at day 11 was less than that when the antibody was added at day 9.

The addition of the type I collagen antibodies did not increase cell death or effect apoptosis (not shown). The overall distribution of mineral in cultures with and without antibodies was not different. The mineral was associated with both thin and thick collagen fibrils and with matrix vesicles whether or not type I collagen was blocked; however, there was substantially less mineral accumulation along the thick collagen fibers when the antibody was present (Fig. 4A, B). Matrix vesicle mineral distribution was not affected by the presence of the collagen antibody (Fig. 4C, D). To verify that the blocking antibodies were having their effect due to interaction with the collagen rather than with forming apatite crystals, solution studies were performed. As summarized in Figure 5, over a wide range of concentrations, neither the nonspecific (Fig. 5A) nor the type I collagen antibodies (Fig. 5B) affected apatite seeded growth. The affinity calculated for IgG binding to apatite based on a Langmuir model was K = 1.44 ml/mg protein, with N = 0.018mg protein/m^2 (Fig. 5C, D).

DISCUSSION

During endochondral ossification in situ, mineral deposits on a matrix containing both types I and II collagens [von der Mark and von der Mark, 1977; Yasui et al., 1984; Bianco et al., 1998]. The variable distribution of these collagen types in the calcifying zones of the growth plates of different species [von der Mark and von der Mark, 1977; Yasui et al., 1984; Reichenberger et al., 1991; Kirsch et al., 1992; Bianco et al., 1998; Ishii et al., 1998; Nie et al., 1999] and the different morphological sites of the same species [Ishii et al., 1998] has led to debate about the importance of type I collagen for cartilage calcification. At least in the chick, there is data demonstrating a genetically controlled switch from type I to type II collagen production in hypertrophic chondrocytes [Boskey and Dick, 1991], however in the chick limb type I mRNA levels are reported to be invariant [Devlin et al., 1988]. The present study, which used a culture system that mimics the initial stages of endochondral ossification, but lacks the osteoclasts responsible for remodeling the tissue, now demonstrates that blocking type I collagen can affect the extent of mineral deposition. The studies also suggest that the immunoblocking procedure is a valid technique for analyzing effects of extracellular matrix proteins on cell-mediated mineralization.

In the differentiating chick limb-bud mesenchymal cell micro-mass culture system, the mesenchymal cells differentiate to form chondrocytes [Binderman et al., 1979; Hadhazy et al., 1982; Boskey et al., 1992a; Shakibaei et al., 1993]. The mesenchymal cells express type I collagen, while the differentiated chondrocytes express type II, IX, XI and later type X collagen [Kosher et al., 1986; Devlin et al., 1988; Boskey et al., 1992a; Daniels et al., 1996; Nah et al., 1996], proteoglycan core protein [Boskey et al., 1992a], and other proteins characteristic of growth plate chondrocytes [Boskey et al., 1992a]. In the chick and rodent micro-mass mesenchymal cell cultures, as well as in the intact animal, Type I collagen expression persists and is expressed in some calcifying nodules along with osteocalcin and bone sialoprotein [Langille and Solursh, 1990] and osteonectin [Pacifici et al., 1990]. The type I collagen may be produced by periochondral cells, fibroblasts, and perhaps by mature hypertrophic chondrocytes [Roach, 1997]. Because the mesenchymal cells that persist in the culture continue to express type I collagen, it has been difficult to use in situ hybridization to conclusively identify the origin of type I collagen in the micromass cultures. It is known that maturation of the cultures is accelerated when cells are grown on a type I collagen substratum, as

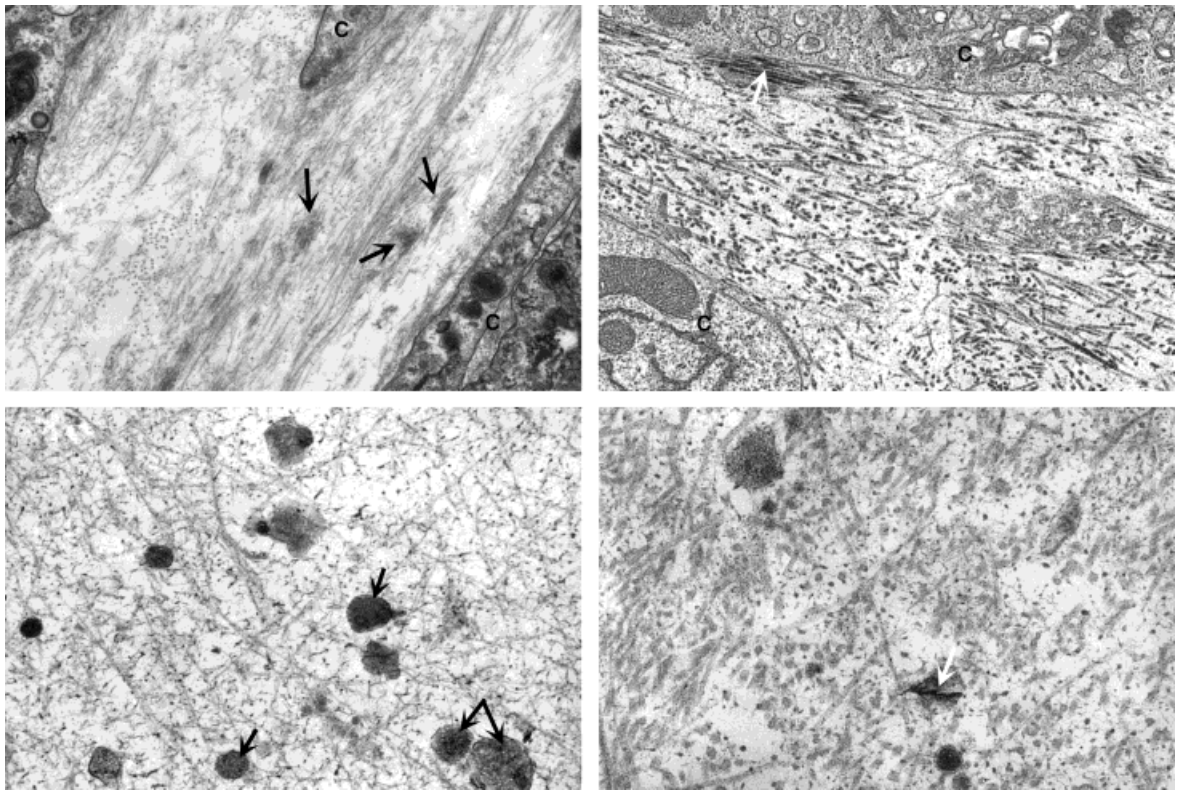

Fig. 4. Appearance of collagen and matrix vesicles in the presence (**A, C**) and absence (**B, D**) of 200 ng/ml collaten type I antibodies, added acutely from day 11. There is much less mineral (arrows) associated with the collagen fibrils adjacent to the chondrocytes (**C**) in the presence of antibody (**A**), than in the absence of antibody (**B**). Magnification 25,000×. In contrast, the distribution of mineral crystals in the extracellular matrix vesicles (arrows) was similar whether or not antibody was present (**C, D**). Magnification 85,000×.

contrasted with laminin fibronectin, or tissue culture plastic [Boskey et al., 1999]. Thus, as has been reported for osteoblasts [Lynch et al., 1995], type I collagen seems to be important for the attachment and differentiation of these cells.

Collagen synthesis is an absolute requirement for mineralization of these cultures [Boskey et al., 1991]. The predominant collagen product of the differentiated mesenchymal cells is type II [Boskey et al., 1992a; Daniels et al., 1996; Langille and Solursh, 1990]. While blocking type II collagen expression was not possible because it is the major product of these cultures, it was hypothesized that blocking antibodies might be used to examine the function of the type I collagen present. Such immunoblocking (neutralizing-antibody) techniques have been used, inter alia, for the in vitro study of thymus cells [Wilson and DeLuca, 1997], breast carcinoma cells [DeLuca et al., 1999] and nerve cells [Wood et al., 1990]. There are few reports of their use in chondrocyte cultures. Two are studies of parathyroid hormone and parathyroid related peptide functions [Harvey et al., 1999; Wood et al., 1990], and there is one study of the use of blocking antibodies to evaluate the role of integrins in the micromass culture system [Shakibaei and Merker, 1999].

Although blocking antibodies have not previously been used in culture systems to test hypothesis about physiologic mineralization, there is a single report that the use of a blocking antibody to bone sialoprotein protein to prevent osteoblast differentiation resulted in a failure of the matrix to calcify [Cooper et al., 1998]. However, because the antibody also decreased osteocalcin and other

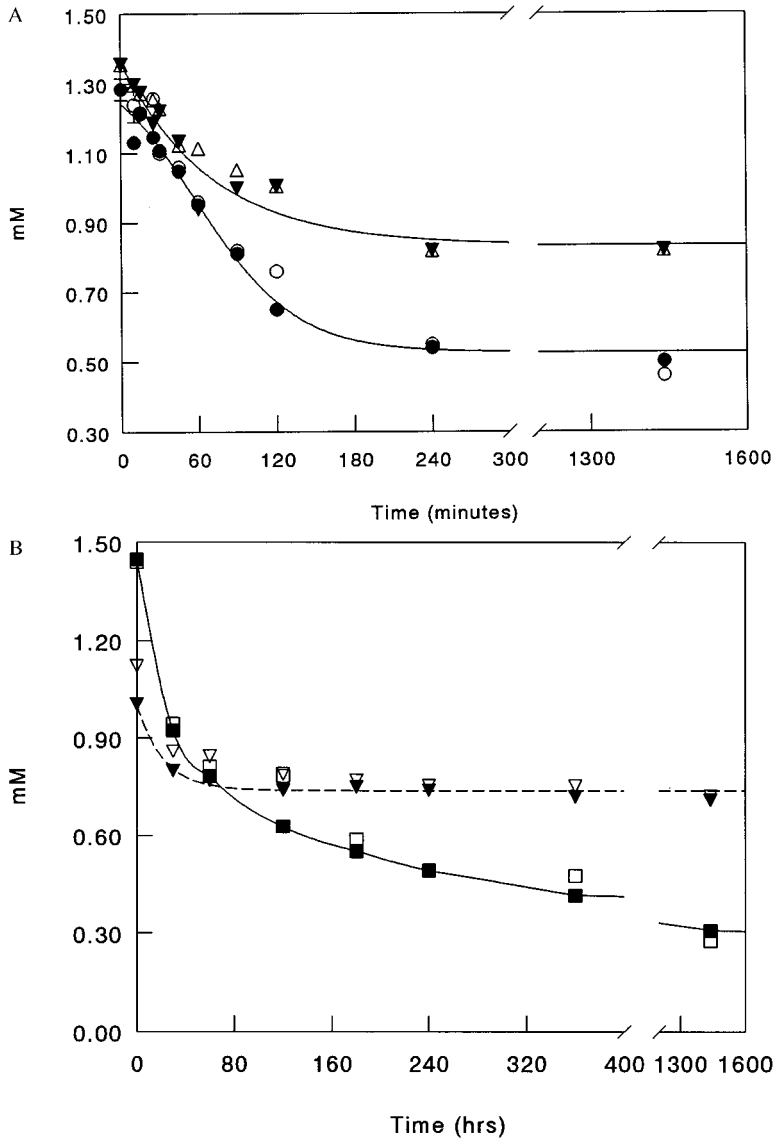

Fig. 5. Effects of Antibodies on Apatite Growth in Cell-Free Solution. **A:** The effect of nonspecific IgG (18 mg/ml) on the growth of apatite seed crystals (0.5 mg/ml). Values shown are Mean ± SD. Concentrations of calcium (squares) and phosphate (triangles) in the presence (open symbols) or absence (solid symbols) of IgG. **B:** The effect of the collagen antibody (200 ng/ml) on the growth of apatite seed crystals (0.5 mg/ml). Symbols are as in 5A. **C:** The binding isotherm for IgG to hydroxyapatite, specific surface 54m2/gm. Ce is the equilibrium concentration of IgG, measured by the BioRad Dye binding assay, Q is the amount bound per surface area of apatite. The insert (**D**) shows the linearization of the data from which the binding constants were determined. The calculated affinity of apatite for IgG was K = 1.44ml/mg protein, N = .018 mg/m^2.

matrix protein accumulation as well as blocking osteoblast differentiation, effects on matrix synthesis and mineralization cannot be distinguished. Results presented in the present study provide evidence that immunoglobulins do not bind significantly to apatite mineral crystals, do not affect mineral crystal growth in vitro, and, therefore, studies with blocking antibodies can be used to evaluate the mineralization process. It must be remembered in these studies, as discussed below, that matrix proteins interact with one another, thus while the current study shows that blocking type I collagen decreases min-

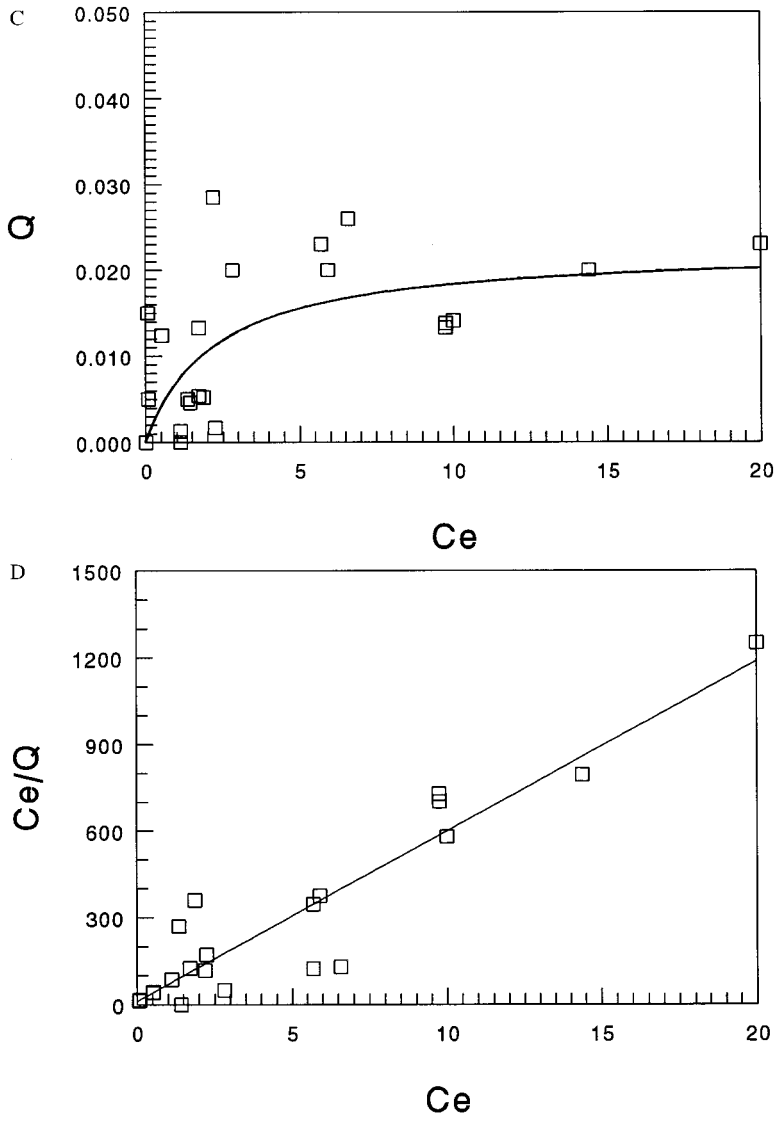

Figure 5. (Continued.)

eral accretion, there are multiple potential mechanisms. It may be that the antibody prevents accumulation of one or more matrix proteins that play a more direct role in collagen-based mineralization, or that the antibody directly prevents the nucleation of calcium phosphate on the collagen fibrils.

When nonspecific antibodies were added to the differentiating mesenchymal cell cultures while cell proliferation and differentiation was dominant (days 2–7), the cultures were reduced in size, and mineral uptake was diminished. This may be attributed to the presence of large molecules in the culture, which in turn might inhibit those cell-cell and cell-matrix interactions that are required for differentiation [Cao et al., 1999]. They may also have blocked access of the cells to nutrients. Addition of nonspecific antibody after day 7, in contrast, had no effect on the appearance of the culture, and on the calcium uptake. Immunoblocking type III collagen, a collagen which is deposited prior to deposition of types I and II collagen in these

cultures [von der Mark and von der Mark, 1977], but which does not mineralize, also did not affect calcium uptake. Immunoblocking of type I collagen on day 9 or 11 did not alter the appearance of the cultures and was not toxic to the cells. It did, however, significantly decrease calcium accumulation. This effect was dose dependent but appeared to saturate since effects with 100 and 200 ng/ml were comparable.

Analogies to in situ mineralization are apparent. During endochondral ossification, as type I collagen is laid down, the extent of mineralization dramatically increases. Although initial mineralization may occur associated with extracellular matrix vesicles [Anderson, 1995], the mineral rapidly spreads to the collagen fibrils. Clinically, altered type I collagen structure in some patients with osteogenesis imperfecta has been associated with defective endochondral ossification noticeable within the calcifying cartilage zone [Cole et al., 1992]. Moreover, in patients with achondrogenesis who lack type II collagen, types I and III collagen is found in their hyaline and growth cartilage enabling them to develop but not mature normally [Chan et al., 1995]. Since type I collagen provides the template for mineral deposition in most tissues throughout the body [Glimcher, 1989], it is reasonable to predict that if sites on the type I collagen fibrils are blocked, mineralization would be decreased. It is possible that the antibodies, both the larger intact ones and the smaller Fab' fragments, block sites near the collagen hole zones and thus decrease initiation of mineral deposition in these sites of initial mineral formation [Glimcher, 1989]. Whether the antibodies are also blocking exposure to noncollagenous matrix proteins that are localized in these zones and can function as apatite nucleators and regulators of apatite growth [Boskey, 1989; Glimcher, 1989] or simply blocking sites on the collagen template, cannot be determined from the current studies.

The success of this immunoblocking study and the lessons learned from it now pave the way for addressing the function of other matrix molecules in the calcification process using the immunoblocking technique. Challenges in the case of the less abundant matrix proteins will be ensuring penetration of the antibody to the sites of matrix protein localization, and selection of the appropriate times for antibody addition. The use of Fab' fragments seems ideal for such analyses.

ACKNOWLEDGMENTS

The authors would like to thank Ms. Lisa Wang and Mr. Alfredo Maldonado for performing the seeded growth studies. Special thanks to Ms. Kitty Bock for preparing the sections for histologic analyses and Ms. Meilin Tan for assisting with the cultures. The authors appreciate the advice of Dr. I. Shapiro on the use of the TUNEL stain.

REFERENCES

Ahrens PBA, Solursh M, Reiter RS. 1977. Stage related capacity for limb chondrogenesis in cell culture. Dev Biol 60:69–82.

Anderson HC. Molecular Biology of extracellular matrix vesicles. 1995. Clin Orthopaed 314: 266–280.

Bianco P, Cancedda FD, Riminucci M, Cancedda R. 1998. Bone formation via cartilage models: the "borderline" chondrocyte. Matrix Biol 17:185–192.

Binderman I, Greene RM, Pennypacker JP. 1979. Calcification of differentiating skeletal mesenchyme in vitro. Science 206:222–225.

Boskey AL. 1989. Noncollagenous matrix proteins and their role in mineralization, bone and mineral. 6:111–123.

Boskey AL, Camacho NP, Mendelsohn R, Doty SB, Binderman I. 1992b. FT-IR Microscopic mappings of early mineralization in chick limb bud mesenchymal cell cultures. Calcif Tissue Int 51:443–448.

Boskey AL, Dick B. 1991. The effect of phosphatidylserine on in vitro hydroxyapatite growth proliferation. Calcif Tissue Int 49:193–196.

Boskey AL, Doty SB, Binderman I. 1999. Adenosine 5' triphosphate promotes mineralization in different chick limb-bud mesenchymal cell cultures. Micros Res Tech 28:492–504.

Boskey AL, Guidon P, Doty SB, Stiner D, Leboy P, Binderman I. 1996. The mechanism of β-glycerophosphate action in mineralizing chick limb-bud mesenchymal cell cultures. JBMR 11:1694–1702.

Boskey AL, Stiner D, Binderman I, Doty SB. 1997. Effects of proteoglycan modification on mineral formation in a differentiating chick limb-bud mesenchymal cell culture system. J Cell Biochem 64:632–643.

Boskey AL, Stiner D, Doty SB, Binderman I. 1991. Requirement of vitamin C for cartilage calcification in a differentiating chick limb-bud mesenchymal cell culture. Bone 12:277–282.

Boskey AL, Stiner D, Leboy P, Doty S, Binderman I. 1992a. Optimal conditions for cartilage calcification in differentiating chick limb-bud mesenchymal cells. Bone and Mineral 14:11–37.

Brackenbury R, Thiery J-P, Rutishauser U, Edelman SM. 1977. Adhesion among neural cells of the chick embryo. J Biol Chem 252:6835–6840.

Cao L, Lee V, Adams ME, Kiani C, Zhang Y, Hu W, Yang BB. 1999. β1-Integrin-collagen interaction reduces chondrocyte apoptosis. Matrix Biol 18:343–355.

Chan D, Cole WG, Chow CW, Mundlos S, Bateman JF. 1995. A COL2A1 mutation in achondrogenesis type II results in the replacement of type II collagen by type I and III collagens in cartilage. J Biol Chem 270:1747–1753.

Cole WG, Patterson E, Bonadio J, Campbell PE, Fortune DW. 1992. The clinicopathological features of three babies with osteogenesis imperfecta resulting from the substitution of glycine by valine in the pro alpha 1 (I) chain of type I procollagen. J Med Genet 29:112–118.

Cooper LF, Yliherikkila, D.A., Felton DA, Whitson SW. 1998. Spatiotemporal assessment of fetal bovine osteoblast culture differentiation indicates a role for BSP in promoting differentiation. J Bone Min Res 13:620–632.

Daniels K, Reiter R, Solursh M. 1996. Micro-mass cultures of limb and other mesenchyme. Methods Cell Biol 51: 237–247.

DeLuca A, Casamassimi A, Selvam MP, Losito S, Ciardiello F, Agrawal S, Salomon DS, Normanno N. 1999. EGF-related peptides are involved in the proliferation and survival of MDA-MB-468 human breast carcinoma cells. Int J Cancer 80:589–594.

Devlin CJ, Brickell PM, Taylor ER, Hornbruch A, Craig RK, Wolpert I. 1988. In situ hybridization reveals differential spatial distribution of mRNAs for type I and type II collagen in chick limb bud. Development 103:111–118.

Edvall-Arvidsson C, Wroblewski J. 1996. Characterization of chondrogenesis in cells isolated form limb buds in mouse. Anat Embryol (Berl) 193:453–461.

Gallagher SR, Smith JA. 1995. Isolation and analysis of proteins, current protocols in immunology, supplement 11:8.4.17–8.4.21.

Glimcher MJ. Mechanisms of calcification in bone: Role of collagen fibrils and collagen-phosphoprotein complexes in vitro and in vivo. 1989. Anat Rec 224:139–153.

Hadhazy C, Lazlo MB, Kostenszky KS. 1982. Cartilage differentiation in micro-mass cultures of chicken limb buds. Acta Morphol Acad Sci Hung 30:65–78.

Hamburger V, Hamilton HL. 1951. A series of normal stages in the development of the chick embryo. J Morphol 88:49–92.

Harvey AK, Yu XP, Frolik CA, Chandrasekhar S. 1999. Parathyroid hormone-(1–34) enhances aggrecan synthesis via an insulin-like growth factor-I pathway. J Biol Chem 274:23249–23255.

Hatori M, Klatte KJ, Teixeira CC, Shapiro IM. 1995. End labeling studies of fragmented DNA in the avian growth plate: Evidence of apoptosis in terminally differentiated chondrocytes. J Bone Mineral Research 10:1960–1968.

Hay DI, Moreno EC. 1979. Differential adsorption and chemical affinities of proteins for apatitic surfaces. J Dent Res 58B:930–942.

Heinonen JK, Lahti RJ. 1981. A new and convenient calorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphates. Anal Biochem, 113:313–317.

Ishii M, Suda N, Tengan T, Suzuki S, Kuroda T. 1998. Immunohistochemical findings type I and type II collagens in prenatal mouse mandibular condylar cartilage compared with tibiial anlage. Arch Oral Biol 43: 545–550.

Kirsch T, von der Mark K. Remodelling of collagen types I, II, and X and calcification of human fetal cartilage. 1992. Bone Miner 18:107–117.

Kosher RA, Kulyk WM, Gay SW. 1986. Collagen gene expression during limb cartilage differentiation. J Cell Biol 102:1151–1156.

Langille RM, Solursh M. 1990. Formation of chondrous and osseous tissues in micro-mass cultures of rat from nasal and mandibular ectomesenchyme. Differentiation 44:197–206.

Lynch MP, Stein JL, Stein GS, Lian JB. 1995. The influence of type I collagen on the development and maintenance of the osteoblast phenotype in primary and passaged rat calvarial osteoblasts: modification of expression of genes supporting cell growth, adhesion, and extracellular matrix mineralization. Exp Cell Res 216:35–45.

Nah HD, Bennett VD, Nui Z, Adams SI. 1996. Alternative transcript of the chick alpha 2(I) collagen gene is transiently expressed during endochondral bone formation and during development of the central nervous system. Dev Dyn 206:146–158.

Nie D, Ishikawa Y, Guo Y, Wu LN, Genge BR, Wuthier RE, Sauer GR. 1999. Inhibition of terminal differentiation and matrix calcification in cultured avian growth plate chondrocytes by Rous sarcoma virus transformation. J Cell Biochem 69:453–462.

Pacifici M, Oshima O, Fisher LW, Young MF, Shapiro IM, Leboy PS. 1990. Changes in osteonectin distribution and levels are associated with mineralization of the chicken tibial growth cartilage. Calcif Tissue Int 47:51–61.

Reichenberger E, Aigner T, von der Mark K, Stoss H, Bertling WK. 1991. In situ hybridization studies on the expression of type X collagen in fetal human cartilage. Dev Biol 148:562–572.

Roach HI. 1997. New aspects of endochondral ossification in the chick: chondrocyte apoptosis, bone formation by former chondrocytes, and acid phosphatase activity in the endochondral bone matrix. J Bone Miner Res 12: 795–805.

Schroter-Kerman C, Hinz N, Risse P, Zimmermann B, Merker HJ. 1991. The extracellular matrix in cartilage organoid culture: biochemical, immunomorphological, and electron microscopic studies. Matrix 11: 428–441.

Shakibaei M, Merker HJ. 1999. Beta1-integrins in the cartilage matrix. Cell Tissue Res 296:565–573.

Shakibaei M, Schroter-Kermani C, Merker HJ. 1993. Matrix changes during long-term cultivation of cartilage (organoid or high-density cultures). Histol Histopathol 8:463–470.

Terkeltaub R, Lotz M, Johnson K, Deng D, Hashimoto S, Goldring MB, Burton D, Deftos LJ. 1998. Parathyroid hormone-related proteins is abundant in osteoarthritic cartilage, and the parathyroid hormone-related protein 1-173 isoform is selectively induced by transforming growth factor beta in articular chondrocytes and suppresses generation of extracellular inorganic pyrophosphate. Arthritis Rheum 41:152–164.

von der Mark K, von der Mark H. 1977. The role of three genetically distinct collagen types in endochondral ossification and calcification of cartilage. J Bone Joint Surg 59B:458–464.

Willis JB. 1960. Determination of metals in blood serum by atomic absorption spectroscopy. I. calcium. Spectrochim Acta 16:259–272.

Wilson SS, DeLuca D. 1997. NOD fetal thymus organ culture: an in vitro model for development of T cells involved in IDDM. J Autoimmun 10:461–472.

Wood PM, Schachner M, Bunge RP. 1990. Inhibition of Schwann cell myelination in vitro by antibody to L1 adhesion molecule. J Neurosci 10:3635–3645.

Yasui N, Ono K, Konomi H, Nagai Y. 1984. Transitions in collagen types during endochondral ossification in human growth cartilage. Clin Orthop 183:215–218.