In Vitro Induction of Cartilage-specific Macromolecules by a Bone Extract

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ABSTRACT An in vitro system has been developed to study the onset of chondrogenesis. Embryonic rat muscle mesenchymal cells, when treated in suspension culture with an extract of bovine bone matrix, synthesized cartilage-specific proteoglycan and type II collagen. The synthesis of these two macromolecules was assayed by the enzyme-linked immunosorbent assay inhibition technique. Further evidence of chondrogenesis was demonstrated by morphological changes of treated cells when cultured in firm agarose and stained for metachromatic matrix. Even with crude bone matrix extracts, the assay was sensitive at the microgram level and significant differences in cartilage macromolecules compared with controls were observed in 2–3 d. In vivo the same extract induced first cartilage and then bone.

Subcutaneous implantation of demineralized bone matrix has been shown to evoke the induction of responding mesenchymal cells into chondroblasts (cartilage-specific cells), an early step in endochrondral bone formation (1–3). It has also been shown that the inducing factor(s) is extractable in stable form with 4 M guanidine·HCl from acid demineralized bone powder (4, 5). Freshly excised skeletal muscle cells from embryonic or neonatal rats cultured on demineralized bone also respond to a cartilage-induction factor (6–8). In monolayer culture, however, chondrogenic development is inhibited by binding of the cells to the plastic culture dish (9, 10). Indeed, the interaction of the cells with a plastic surface appears to promote the dedifferentiation of chondrogenic cells into cells with a fibroblastic morphology that no longer synthesize cartilage proteoglycan and type II collagen (11). However, Anastassiades et al. (9) showed that a 4-M guanidine·HCl extracted, saline-soluble fraction prepared from bone matrix increases glycosaminoglycan secretion by cultured fibroblasts.

We have expanded on these observations and developed a rapid, sensitive in vitro system for induction of cartilage synthesis in response to solubilized bovine bone chondrogenic factor(s). Specificity and quantitation was obtained by employing antibodies to rat cartilage proteoglycan and rat type II collagen.

MATERIALS AND METHODS

Tissue Preparation and Extraction: Fresh metatarsal bovine bone were frozen overnight at −70°C. Frozen bone was cleaned of periosteum, broken into fragments, and pulverized in liquid nitrogen. Pulverized bone was washed overnight in 0.01 N HCl at 4°C and defatted with ethanol and ether as described elsewhere (3). Bone powder was demineralized with 50 vol (wt/vol) of 0.5 N HCl at 4°C for 16 h. Acid was removed and pellet was washed with distilled water to pH 4. Demineralized bone was extracted with 10 vol (vol/vol) of 4 M guanidine·HCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 mM *N*-ethylmaleimide, pH 6.5 for 16 h at 4°C. The suspension was centrifuged at 10,000 rpm for 30 min. The pellet was washed with another 5 vol of the 4-M guanidine·HCl solution and centrifuged as above. Supernatants were combined, dialyzed extensively against water, and lyophilized.

Ion Exchange Fractionation: An anionic exchanger, DEAE-cellulose (Whatman DE-52; Whatman Chemicals, Kent, England), equilibrated with 6 M urea, 1 mM phenylmethylsulfonyl fluoride, 1 mM *N*-ethylmaleimide, 20 mM phosphate (made from stock solution of 0.15 M Na_2HPO_4, 0.15 M NaH_2PO_4) and 50 mM NaCl adjusted to pH 7.2 with appropriate 0.15 M phosphate solution. The lyophilized guanidine·HCl extract dissolved in DEAE-cellulose equilibration buffer was mixed with DEAE-cellulose (20 mg of dry extract per gram of DEAE) that had been equilibrated with the same buffer and stirred gently over a period of 1–2 h at room temperature. The nonbound fraction, which constituted ~50% (dry weight) of the material applied to the column, was recovered by filtration. The bound fraction was eluted by increasing the NaCl concentration to 1 M in starting buffer. For in vivo studies, nonbound and bound fractions were dialyzed extensively against distilled water, lyophilized, rehydrated with 1 vol of distilled water, and dehydrated with ethanol followed by ether to make a coarse powder. For in vitro assays, these fractions were solubilized in 6 M urea sterilized by filtration (0.2-μm Millipore filter; Millipore Corp., Bedford, MA) and dialyzed extensively against sterile water followed by sterile PBS.

Isolation of Cells: Embryonic rat muscle cells were obtained from the cellular outgrowth of minced muscle tissue dissected from the upper limbs of 19-d-old Sprague-Dawley rat fetuses. Tissue and cells were cultured in Minimum Essential Medium with 10% fetal calf serum, 50 U penicillin, and 50 μg streptomycin per ml. At confluence, cells were trypsinized and diluted 1:2 for replating. Cells were used for experiments within the first three passages. Except where noted, culture solutions and sera were obtained from GIBCO Laboratories, Grand Island, NY.

Cell Culture: Cells were grown in monolayer or in suspension culture. Suspension cultures were established by growing the cells on bacteriological plates, poly-D-lysine (Sigma Chemical Co., St. Louis, MO) treated plates (see below) or on agarose (Bio-Rad Laboratories, Richmond, CA; #162-0100). Tissue culture plates (35 mm, Costar, Cambridge, MA) were treated with poly-D-lysine by coating with 1 ml of a 1 mg/ml aqueous solution. The plates were

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then allowed to dry; they were rinsed and dried three times subsequently. Plates treated in this manner were shown to prevent significant cell attachment in a manner resembling bacteriological plates. For assay, cells were seeded at 5×10^5 cells per 35-mm well in Hams F-12 medium containing 10% fetal calf serum and a weighed portion of the dry bone extract. Parallel cultures were also established which included 50 μg/ml β-aminoproprionitrile as a collagen cross-linking inhibitor (12). Viable cells were counted in a hemocytometer using trypan blue.

Preparation of Agarose Gel Cultures: Cells were embedded in agarose gels according to the method of Benya and Shaffer (11). Briefly, 0.15 ml of 1% agarose (Bio-Rad Laboratories, #162-0100) heated above 90°C was added to each 17-mm well (Falcon Labware, Oxnard, CA) and allowed to gel on a level surface at room temperature. Low gel melting agarose (Bio-Rad Laboratories, #162-0017), 2% at 38°C, was mixed with an equal volume of two times concentrated Ham's F-12 and held at 38°C. The agarose/F-12 solution was then mixed with an equal volume of cell suspension and 0.15 ml was added to each well. When firm, gels were overlaid with 1.0 ml of media, cultured at 37°C in 5% CO_2, and fed twice weekly. Media for cell feeding consisted of Ham's F-12 with 10% heat-inactivated fetal calf serum, and antibiotics. Agarose solutions were sterilized by autoclaving at 121°C for 15 min prior to use.

Staining of Agarose Gel Cultures for Metachromasia: After 14 d of culture, gels were fixed and stained with Toluidine blue (Sigma Chemical Co.) as described by Horwitz and Dorfman (13). Gels were briefly rinsed twice in PBS, fixed in 40% formalin, rinsed twice with distilled water, stained with 0.5% Toluidine blue in 25% acetone, and washed with distilled water.

Antisera: Antisera to rat cartilage proteoglycan and type II collagen were raised in rabbits. Both antigens were extracted from Swarm rat chondrosarcoma tissue (14). Type II collagen was isolated from a 1 M NaCl extract of sarcoma tissue followed by precipitation with 0.02 M sodium phosphate (16). Proteoglycans were eluted from a DEAE-cellulose column with 2 M NaCl (16), followed by gel filtration on Sephadex G-200 (15). Elution was monitored by uronic acid (25). Antigens were dissolved in PBS at 1 mg/ml and were mixed with an equal volume of complete Freund's adjuvant prior to subcutaneous injection. Four weeks later animals were boosted with 1 mg antigen in incomplete adjuvant. Boosters were given two more times at 2-wk intervals. Significant titers were obtained from sera collected following the third and fourth injections. Antiserum to type II collagen was affinity purified (17), employing highly purified type II collagen from the chondrosarcoma carried in lathyritic rats (14) and shown not to cross-react with type I collagen. In the experiments reported here antiserum to proteoglycan was not affinity purified. However, in selected experiments using affinity purified antibodies identical results were obtained. Cross reactivity of cartilage proteoglycan antiserum with bone proteoglycan, hyaluronic acid, and the bone extract was found to be negligible. Treatment of proteoglycan with chondroitinase ABC had little effect on antigenicity; this suggests that the antiserum is directed primarily against core protein or oligosaccharides attached to core protein.

Enzyme-linked Immunosorbent Assay: Synthesis of proteoglycan and type II collagen was measured directly in culture media. Media samples were collected at the indicated times and stored at −70°C. Enzyme-linked immunosorbent assay was performed essentially as described by Rennard et al. (18) using polystyrene microplates (Flow Laboratories, Inc., McLean, VA). Horseradish peroxidase conjugated goat anti-rabbit IgG (Tago Inc., Burlingame, CA) was used as the second antibody. Samples were either assayed directly or diluted in PBS, 0.05% Tween 20, 1 mg/ml BSA and quantitated by use of an inhibition enzyme-linked immunosorbent assay (19) using chondrosarcoma proteoglycan or type II collagen as a standard.

RESULTS

In vivo experiments with 4 M guanidine·HCl soluble material extracted from rat demineralized bone and fractionated on DEAE-cellulose have shown that only the nonbound material induces cartilage and bone formation when implanted in rats (5). Similarly, we found the equivalent fraction from bovine demineralized bone to be a potent inducer of chondrogenesis and osteogenesis when implanted subcutaneously in rats. Activity was not detected in material bound to DEAE-cellulose (data not shown).

In the present study, rat muscle mesenchymal cells grown in suspension culture and treated with the DEAE-cellulose nonbound fraction of bone extract underwent dramatic biochemical changes. As shown in Fig. 1, cells grown in suspension on bacteriological plates, poly-D-lysine coated plates, or

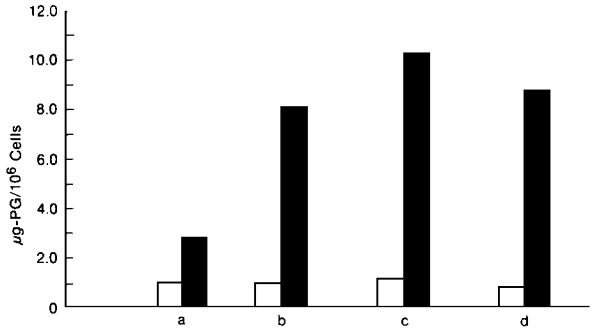

FIGURE 1 Synthesis of cartilage proteoglycan (*PG*) by cells cultured in *a*, monolayer; *b*, poly-D-lysine coated plates; *c*, bacteriological plates; *d*, agarose coated plates. Cells were seeded at 5×10^5 cells per well in the presence (solid bars) or absence (open bars) of 20 g/ml of DEAE-cellulose nonbound extract. Media were collected after 7 d in culture and tested for cartilage proteoglycan enzyme-linked immunosorbent assay.

on agarose responded best to the DEAE-cellulose nonbound extract. Cells in suspension in the presence of extract synthesized 8–10 times as much proteoglycan as cells in suspension in the absence of extract, and 3–4 times as much as cells in monolayer in the presence of extract. Response to the active extract was dose dependent; synthesis of type II collagen and proteoglycan was detected with 5 μg/ml and continued to increase up to 50 μg/ml of DEAE-cellulose nonbound material in the culture media.

There was a significant increase in cartilage proteoglycan synthesis (Fig. 2*A*) as well as type II collagen synthesis (Fig. 2*B*) when cells were cultured with 20 μg/ml DEAE-cellulose nonbound material on poly-D-lysine coated culture plates. In both cases, increases in the amount of the cartilage markers could be clearly seen as early as 2 d after treatment with a further increase at 3 d. The collagen cross-linking inhibitor β-APN has been used extensively in cell culture systems to increase the proportion of matrix molecules released into the culture medium (20, 21). This compound was effective in our experiments in increasing the amounts of both type II collagen and proteoglycan in the medium. Although not quantitated, these macromolecules were detected in both the cell layer and the matrix by immunofluorescence. Control experiments with rat chondrocyte cultures gave similar results showing the specificity of the test system.

Agarose gel culture has been shown to be permissive to the chondrocyte phenotype as evidenced by type II collagen and cartilage proteoglycan synthesis (11). To confirm morphologically the chondrogenic phenotype of cells treated with the DEAE-cellulose nonbound fraction in suspension, they were embedded in agarose gels. After 14 d in agarose gels, cells treated with active extract for the first 7 d showed large colonies; and substantial numbers of these cells had the grainy refractile appearance typical of chondroblasts (Fig. 3*A*). Cells treated with the DEAE-cellulose bound fraction, on the other hand, contained many degenerated cells and few large colonies (Fig. 3*B*). When stained with Toluidine blue (not shown), metachromatic halos, indicative of a proteoglycan matrix, appeared around a majority of cells treated with the DEAE-cellulose nonbound fraction. Cells treated with the DEAE-cellulose bound fraction or untreated, showed no metachromasia. Immunobiochemical analysis of macromolecules extracted from these gels revealed the synthesis of cartilage proteoglycan and type II collagen in the presence of active

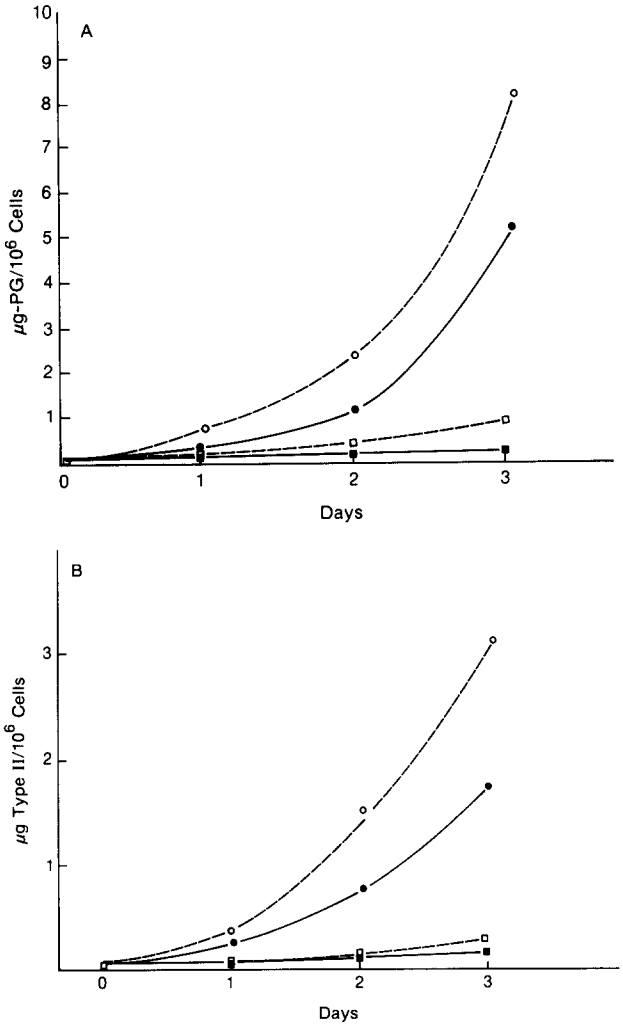

FIGURE 2 Synthesis of cartilage biochemical markers by mesenchymal cells cultured with DEAE-cellulose nonbound extract. Cells were cultured on poly-D-lysine coated plates in Hams F-12 medium, 10% FCS, in the presence (circles) or absence (squares) of 20 g/ml dry extract. Parallel cultures were incubated in the presence of 50 g/ml β-aminoproprionitrile (open symbols). Media were collected at the indicated times and assayed for proteoglycan (*PG*) synthesis (*A*) or type II collagen synthesis (*B*). Values are normalized for cell number.

extract in the same manner as shown for cells grown on poly-D-lysine coated plates (Fig. 2).

DISCUSSION

The increases in cartilage proteoglycan and type II collagen synthesis observed in vitro in our studies resemble those observed in vivo after implantation of demineralized bone matrix (22). In both instances, increases in cartilage proteoglycan synthesis can be detected as early as 2–3 d after exposure to active material. The in vitro system is of course simpler, more sensitive, and easily quantitated.

Although it is well known that bone extracts contain mitogenic factors (23, 24), the finding that significant increases in type II collagen and proteoglycan synthesis occurred as soon as 2 d after treatment argues against cell division being an important factor. In confirmation, no significant differences in viable cell numbers were seen in cultures treated with DEAE-cellulose nonbound or bound fractions, or in control

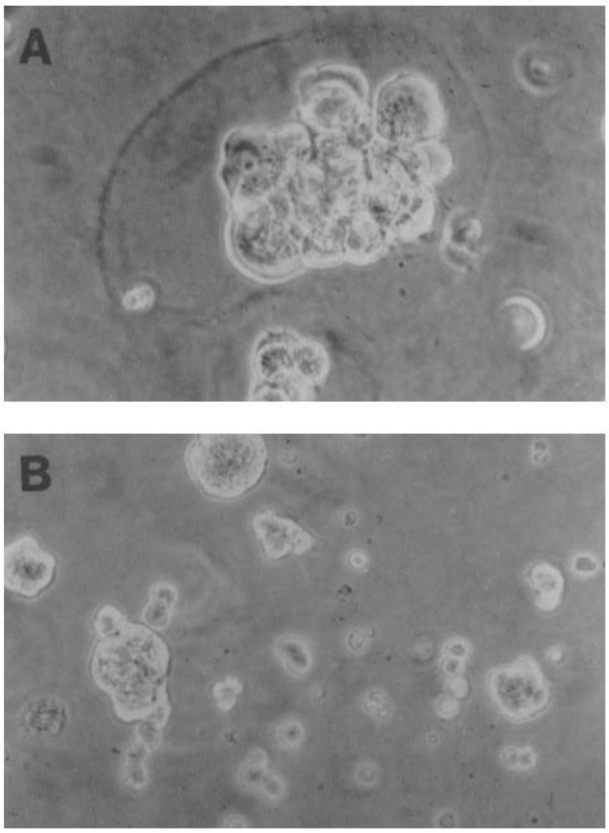

FIGURE 3 (*A*) Cells treated with DEAE-cellulose nonbound fraction in suspension (poly-D-lysine coated plate) for 7 d and embedded in agarose for 14 d. Cells are large, form clusters, and have the grainy appearance of actively synthesizing cells. (*B*) As in *A* but treated with DEAE-cellulose bound fraction. Cells form some clusters but do not increase in size. Control (untreated) cells appeared identical to these. × 200.

cultures after 3 d on poly-D-lysine coated or bacteriological plates. Similarly, cells treated with active extract showed no increase in DNA synthesis over controls as demonstrated by [^{3}H]thymidine incorporation studies (in preparation).

Although the mechanism of action of the bone extract is not known, the significant changes in cell morphology and biochemical markers suggest that as yet unidentified muscle-derived mesenchymal cells undergo a true change in phenotype. The development of a rapid and sensitive in vitro system should be an important tool in the purification and characterization of chondroblast inducing factor(s), and might be an avenue to study the mechanism of endochondral bone formation as induced by demineralized bone matrix factor(s).

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