Journal of Cellular Biochemistry 65:325–339 (1997)

Combination of Osteoinductive Bone Proteins Differentiates Mesenchymal C3H/10T1/2 Cells Specifically to the Cartilage Lineage

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Abstract During embryonic development, cartilage formation involves the condensation of mesenchymal stem cells and a series of maturation steps that ultimately results in the mineralized hypertrophic chondrocyte. The embryonic, murine, mesenchymal stem cell line, C3H/10T1/2, is pluripotent; exposure to azacytidine or to bone morphogenetic protein-2 or -4 results in low rates of differentiation to three mesengenic lineages. In contrast to previous studies, we report conditions for 10T1/2 differentiation specifically to the cartilage lineage and at high yields. These conditions include high cell density micromass cultures, a purified mixture of osteoinductive proteins (BP; Intermedics Orthopedics, Denver, CO), a serum substitute, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate. The cartilagenous fate was confirmed by 1) histological detection of sulfated proteoglycans, 2) electron microscopic detection of proteoglycan and rounded cells separated by extracellular matrix containing short, disorganized collagen fibrils, 3) morphological detection of chondrocytes surrounded by a territorial matrix and encompassed within a distinct perichondrium, and 4) immunocytochemical detection of type II collagen and link protein. After 4 weeks in culture, mature although unmineralized cartilage was observed, as indicated by hypertrophic morphology, immunocytochemical detection of osteocalcin, and histological detection of lacunae. These conditions promote overt chondrogenesis for most of the treated cells and preclude lineage determination to the fat, muscle, and bone lineages, as assayed by electron microscopy and histomorphology. The faithful recapitulation of cartilage differentiation that we have established in vitro provides a versatile alternative to the use of chondrocyte and limb bud explant cultures. We propose this as a model system to study the factors that regulate commitment to the chondrogenic lineage, exclusion to related mesengenic pathways, and maturation during chondrogenesis. J. Cell. Biochem. 65:325–339.

Key words: bone morphogenetic protein; defined media; in vitro; development; stem cell; ascorbic acid

During embryonic development, pluripotent stem cells differentiate to specific cell fates. In this process, stem cells must commit to a specific lineage and repress lineage determination to alternative pathways. Chondrogenesis is a multistep pathway that involves differentiation of mesenchymal stem cells, followed by expression of cartilage-specific genes producing extracellular matrix proteins, and maturation of prechondrocytic cells into hypertrophic chondrocytes. The factors that regulate this process include growth factors, extracellular matrix proteins, cell-cell juxtaposition, and specific transcription factors that regulate the composition of cartilage as well as many unidentified factors [Erlebacher et al., 1995]. One pivotal stage regulating prechondrogenic skeletal development is cell condensation, which results from epithelial-mesenchymal interactions and leads to cell lineage determination [Hall and Miyake, 1992].

The cytokines involved in bone and cartilage development have been extensively studied. Ini-

Contract grant sponsors: Colorado Institute for Research in Biotechnology, Intermedics Orthopedics, Inc., Lucille P. Markey Charitable Trust.

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Received 22 March 1996; accepted 16 November 1996

tially, demineralized bone matrix was found to induce ectopic bone formation when implanted subcutaneously or intramuscularly in rodents (ectopic assay system). This bone-forming activity resulted from factors termed bone morphogenetic proteins (BMPs) [Urist, 1965; Urist et al., 1973], and this ectopic assay was used to monitor BMP purification. Seven BMPs were cloned, six of which belong to the transforming growth factor beta (TGFβ) superfamily of cytokines [Wozney, 1993]. In the ectopic assay system, BMP implants first induce cartilage formation, followed by cartilage maturation, removal of cartilage, and replacement with bone. The sequence of events simulates endochondral bone formation. Similarly, demineralized bone matrix was found to contain cartilage inducing factor-A and -B, which were subsequently identified as TGFβ-1 and TGFβ-2 [Seyedin et al., 1986, 1987]. TGFβ-1 has been shown, both in vivo and in vitro, to enhance chondrogenesis but is unable to induce bone formation in the ectopic assay system [Denker et al., 1995; Izumi et al., 1992; Joyce et al., 1990; Kulyk et al., 1989; Miura et al., 1993; Wozney et al., 1988].

An in vitro model of differentiation from mesenchyme to cartilage would facilitate understanding of the molecular steps involved in cartilage development. Murine, mesenchymal 10T1/2 cells are multipotential and were originally found to differentiate into the three distinct lineages of muscle, fat, and cartilage upon 5-azacytidine addition [Taylor and Jones, 1979]. Cartilage cells were detected in 0.1–1% of the total number of cells, but the majority (25–50%) of cells differentiated to the muscle lineage. Similarly, stable 10T1/2 transfectants that express BMP-2 or BMP-4 in confluent monolayer cultures induced differentiation to the three lineages of bone, fat, and cartilage [Ahrens et al., 1993]. Only 0.2% of the cells differentiated to these lineages for the BMP-2 construct, and, of those, chondrogenic cells were represented at 5- to 25-fold fewer cells than cells of the osteogenic or adipogenic lineages. 10T1/2 cells expressing BMP-4 promoted differentiation primarily to the adipogenic lineage. Exogenous addition of BMP-2 to confluent cultures of 10T1/2 cells produced a similar effect as stable BMP-2 transfectants [Katagiri et al., 1990; Wang et al., 1993]. In contrast, the chondrogenic pathway was favored when 10T1/2 cells were plated in high density micromass cultures, which mimics the in vivo cellular mesenchymal precursor stage of chondrogenesis [Ahrens et al., 1977]. 10T1/2 micromass cultures treated with TGFβ-1 induced differentiation to a precartilagenous condensation stage, whereas the myogenic pathway was not detected [Denker et al., 1995].

The evidence using 10T1/2 cells suggests that both the cellular environment and the particular growth factor is critical for cartilage fate determination in vitro. More importantly, these data suggest that the 10T1/2 micromass system can be employed as a model for chondrogenesis. However, these studies have failed to 1) optimize conditions for chondrogenic maturation, including favorable media environment and a growth factor mixture, 2) attain high rates of differentiation to the cartilage lineage, 3) progress through stages of chondrogenic maturation, ultimately to individual chondrocytes, 4) exclude lineage determination to related mesengenic pathways, and 5) accurately describe the chondrogenic or osteogenic stages of development due to lack of morphological and electron microscopy analysis.

To optimize conditions of chondrogenesis and to more fully mimic in vivo chondrogenic conditions, we employed micromass cultures of mesenchymal 10T1/2 cells and exposed them to a fraction of demineralized bone matrix (BP) that has been purified on the basis of cartilage and bone formation in the ectopic assay system (Intermedics Orthopedics, Inc., Denver, CO). We extensively characterized differentiation of mesenchymal 10T1/2 cells by morphology, histology, immunocytochemistry, and electron microscopic analysis. We report conditions for the optimization of chondrogenesis in which the majority of mesenchymal cells differentiates to a cartilage fate in a stage-specific manner over time, culminating with individual chondrocytes surrounded by a territorial matrix. In addition, differentiation to the related fat, bone, and muscle pathways is excluded. Since this in vitro model mimics in vivo cartilage differentiation, it provides a powerful system to elucidate the molecular and cellular controls governing the chondrogenic developmental pathway.

METHODS

Cell Culture

C3H10T1/2 clone 8 cells were obtained from the American Type Tissue Collection (Rockville, MD) and maintained on 100 × 20 mm standard plates (Becton Dickinson, San Jose,

CA) in Dulbecco's modified Eagle's Medium (DMEM) plus 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 mg/ml streptomycin. Cultures were incubated in a humidified incubator at 37°C and 5% CO_2. Only passages 8–18 were utilized for these studies.

The micromass culture technique was performed as described previously with minor alterations [Ahrens et al., 1977; Denker et al., 1995]. Briefly, trypsinized cells were resuspended in DMEM + 10% FBS at a concentration of 10^7 cells/ml, and 10 µl of cells were placed in the center of a well of a 24-well polystyrene microtiter tissue culture dish (Becton Dickinson). After 2–3 h at 37°C, 1 ml of DMEM containing either 10% FBS (FBS) or 1% Nutridoma-SP (Boehringer-Mannheim, Indianapolis, IN) and BP was added. In cultures containing 10% FBS, the media was removed after 3 days and replaced with fresh media lacking BP, which was replaced every other day thereafter. In cultures containing Nutridoma, the media was replaced approximately every 7–14 days with Nutridoma lacking BP.

BMP-2 and BP

Human recombinant BMP-2 and BP (bone protein) were generous gifts from Genetics Institute (Cambridge, MA) and J. Benedict (Intermedics Orthopedics, Inc., Denver), respectively. The protocol for BP purification (described in US patent 5,290,763) is summarized as follows. Cortical diaphyses from bovine long bones were cleaned of marrow and soft tissues, pulverized, sized, and demineralized in 1 M HCl. The demineralized bone matrix particles were washed with water and extracted with a 4 M guanidine solution, pH 7.4, at 15°C for 48 h. The extracted proteins were ultrafiltered through 100 K and 10 K cutoff ultrafiltration membranes to enrich for the 10–100 K protein fraction. Additional purification was achieved using anion and cation exchange chromatography and by reversed phase HPLC. During purification, osteoinductive activity was confirmed using the ectopic assay system. In this assay, a bovine type I collagen sponge disc containing BP or a control lacking BP was implanted subcutaneously in rats for 21 days. Histological analysis of the cells migrating into the disc revealed mature bone of osteoblastic origin and abundant hematopoietic marrow only in BP-containing discs and not in control discs. Although the precise composition of BP is yet to be defined, its osteoinductive activity in vivo, its polyacrylamide gel electrophoresis banding patterns, and the purification protocol are consistent with those of other osteoinductive protein preparations, such as those containing the bone morphogenetic proteins. Lyophilized BP was dissolved in 1 mM HCl prior to use and diluted in media at final concentrations of 0.02–12.5 µg/ml. BP was omitted in the weekly media changes.

Histology

Spheroids were dehydrated and fixed for 20 min in absolute methanol at 4°C. Fixed sections were infiltrated and polymerized using the glycol methacrylate embedding technique as previously described [Block et al., 1982]. The polymerized plugs were then sectioned at 5 mm thickness using a JB-4 Sorvall (Wilmington, DE) microtome. Sections were mounted on silane-coated slides and stained with 0.2% Azure II at pH 1.

Polyclonal and Monoclonal Antibodies

Polyclonal goat primary antibodies were obtained from Southern Biotechnology Associates Incorporated (Birmingham, AL) for type I, II, and III collagens and from Biomedical Technologies Incorporated (Brighton, MA) for osteocalcin. The antibodies against the collagens and osteocalcin were diluted 1:200 and 1:5, respectively, in 0.5 M Tris-HCl (pH 7.4), 1% BSA, and 1% Sodium Azide prior to use. Negative control slides for these antibodies were incubated with 1:200 normal goat serum instead of the primary antibody. Monoclonal mouse primary antibodies for osteopontin, bone sialoprotein, and link protein were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA) and were not diluted prior to use. Negative control slides were incubated for these antibodies with 1:200 nonimmune mouse serum instead of the primary antibody.

Immunocytochemistry

Spheroids were snap-frozen in a 100% isopentane/dry-ice solution, sectioned at 5 µm thickness using a Reichert-Jung cryostat, and mounted on silane-coated slides. Frozen sections were then fixed in 1% paraformaldehyde (EM grade; Electron Microscopy Sciences, Ft. Washington, PA) in 0.4 M EDTA (pH 7.4) for 20 min, rinsed in 0.05 M Tris-Cl (pH 7.4), blocked with 1% BSA for 20 min at room temperature, and incubated with either goat or mouse pri-

mary antibody for 1 h at room temperature. After rinsing, the sections were blocked with 10% normal rabbit serum for 20 min at room temperature. The sections were then treated with a 1:2,000 biotinylated, rabbit antigoat IgG (Gibco-BRL, Gaithersburg, MD) followed by an incubation with a 1:100 streptavidin-conjugated alkaline phosphatase (Dako Corp., Carpinteria, CA). Each incubation was for 30 min at room temperature. The mouse-antibody-treated slides were incubated with an unlabeled rabbit antimouse (rat absorbed) antibody (Dako Corp.) and then incubated with an alkaline phosphatase antialkaline phosphatase antibody (a generous gift of Dr. Wilbur Franklin, UCHSC, Denver, CO). Each incubation was for 30 min at room temperature. The reaction was visualized with an alkaline phosphatase substrate, New Fuchsin, as previously described [Stein et al., 1985]. The slides were counterstained with Gill's #1 hematoxylin for 10 s, dehydrated through graded alcohols, and cleared in Americlear xylene substitute.

Electron Microscopy

Electron microscopy was performed as described previously [Miller and Jones, 1987].

RESULTS

BP Does Not Induce Adipocyte Differentiation in 10T1/2 Monolayers

Previous studies have demonstrated that murine mesenchymal 10T1/2 monolayers cultures treated with BMP-2 or BMP-4 and grown to confluence induce the characteristic adipocyte morphology of large, lipid vescicles contained within the cellular cytoplasm [Ahrens et al., 1993; Wang et al., 1993]. To determine whether BP also induced adipogenesis, low cell density 10T1/2 monolayer cultures were treated with BMP-2 or BP and assayed for appearance of adipocyte morphology in DMEM media containing 10% FBS (Fig. 1). After confluence, adipocytes were clearly observed in BMP-2 (1 μg/ml)–treated cultures (Fig. 1A). In contrast, no adipocyte morphology was observed in untreated or BP-treated (1 μg/ml) cultures (Fig. 1B,C). Also, note that the majority of BMP-2– and BP-treated cells are spindle-shaped and are overlapping, but untreated cells exhibit the characteristic contact-inhibited fibroblast morphology (Fig. 1). One caveat to these studies is that BP is antimitogenic compared to BMP-2–treated or untreated cultures in the presence of FBS, which may result in a lower cell density.

As an initial attempt to induce chondrogenesis, 10T1/2 micromass cultures were induced to differentiate with TGFβ-1 in the presence of FBS, as previously described [Ahrens et al., 1977; Denker et al., 1995]. The micromass underwent a series of events including condensation and formation into a three-dimensional, sphere-like structure, termed a spheroid [Denker et al., 1995]. Under these conditions, BP induced a very similar progression of stages,

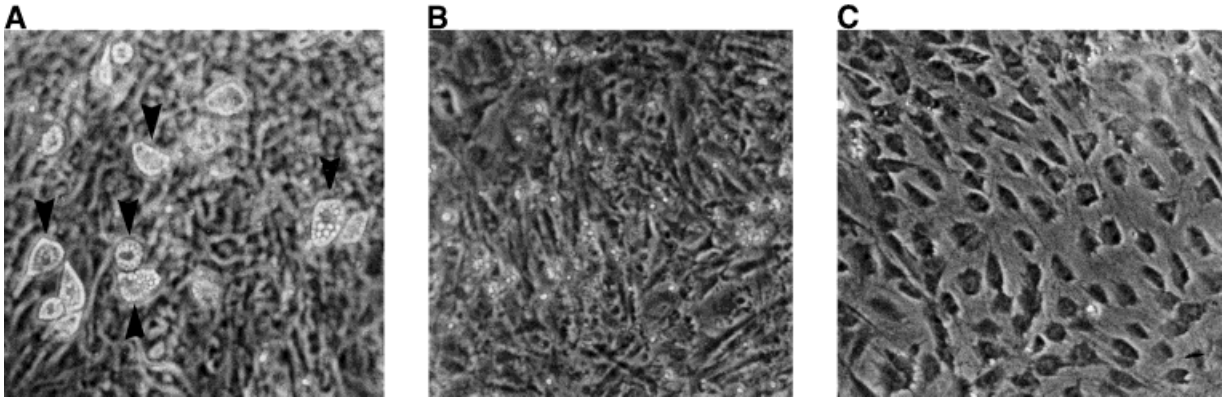

Fig. 1. BMP-2 induced adipocyte morphology, but BP did not. Two thousand 10T1/2 cells were seeded, in triplicate, in 35 mm dishes containing DMEM/10% FBS and treated with 10, 100, or 1,000 μg/ml BMP-2 or 10, 100, or 1,000 μg/ml BP. The cultures were analyzed over a period of 18 days. After reaching confluence, adipocyte morphology was observed for cultures treated with 1 μg/ml BMP-2 (**A,** *arrowheads*). Fewer adipocytes were observed in cultures treated with 10 and 100 μg/ml BMP-2. Adipocytes were not observed in cultures treated with 1 μg/ml BP (**B**) or untreated cultures (**C**). However, low quantities of small, refractile lipid vescles were observed in untreated and BP-treated cultures, which are not characteristic of adipocytes (B,C). ×25.

which also culminated in spheroid formation (not shown).

Serum Substitute Enhances BP-Induced Chondrogenesis

Chondrogenic differentiation in vitro can be enhanced with low serum media and supplementation with insulin and transferrin [Bohme et al., 1992; Cancedda et al., 1992; Goldring et al., 1994; Kato et al., 1980; Quarto et al., 1992; Stevens et al., 1981; Takigawa et al., 1991]. Figure 2 shows that BP induced spheroid formation in 1% Nutridoma-SP (contains insulin and transferrin) media. BP induced four morphologically distinct stages over a period of 2 days. In stage I, no difference in micromass morphology is observed between micromasses treated with BP and those untreated. The micromass periphery consisted of low density, fibroblast cells, whereas the inner core consisted of a high density of cells in multiple layers; no particular cellular organization within the micromass was observed at this time. Stage II is observed only in media containing 10% FBS and is described below. In stage III, treated cells condense to form a smaller, more compact micromass, which lifts off the plastic as a two-dimensional sheet structure (stage IV) and forms a three-dimensional spheroid (stage V). From each micromass, only a single spheroid (approximately 2–3 mm in diameter) forms. A ring of fibroblastic cells remains attached to the plate coinciding with the outside periphery of cells that did not condense in stage III. Untreated micromasses did not progress through the morphological changes characteristic of BP-treated micromasses (Fig. 2A). Instead, individual cells became removed from the plate, rounded, and refractile, characteristics indicative of cell death caused by the lack of growth factors in the media (Fig. 2A, stage V). To determine whether this phenomenon occurs with any micromass cell culture, we treated the cervical epithelial HeLa cell line with BP. HeLa micromasses in the presence or absence of these factors remain in stage I (not shown).

Comparisons of micromass cultures grown in Nutridoma or in FBS revealed three phenomenological differences. First, spheroids appeared more quickly in Nutridoma, in as few as 24 h rather than 3 days that is typically required in FBS conditions. Second, formation of spheroids was independent of media change. Micromass cultures grown in FBS often did not form spheroids if the media was not changed every other day. Third, micromass cultures containing Nu-

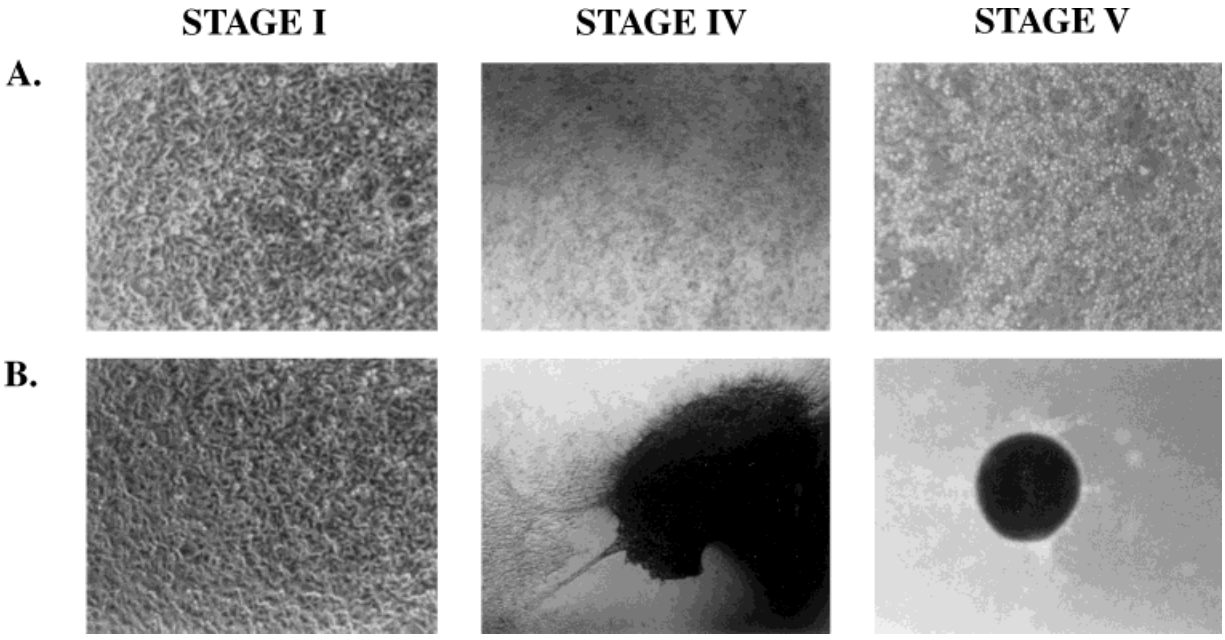

Fig. 2. Morphology of murine 10T1/2 micromass cultures in the absence and presence of BP. 10T1/2 micromass cultures were induced to differentiate with 1% Nutridoma only (**A**) or Nutridoma containing 0.5 µg/ml BP (**B**). Stages I and IV are 4 h and 2 days after induction to differentiate, respectively. Stage V is 6 (A) and 11 (B) days after induction to differentiate. Stage I: ×25. Stages IV, V: ×10.

tridoma did not undergo a stage II palisading phenomenon that is observed with FBS: cells at the periphery of the micromass become elongated and bipolar and align in parallel arrays.

To compare the extent of chondrogenesis in Nutridoma and FBS conditions, 10T1/2 micromass cultures were treated with 0.5 μg/ml BP in either medium, and the resulting spheroids were harvested at day 7 and stained with Azure at pH 1. Cells within Nutridoma-grown spheroids were rounded and separated by extensive regions of diffuse, metachromatically stained extracellular matrix, indicative of a chondrogenic stage of differentiation (Fig. 3B) [Lev and Spicer, 1964; Schubert and Hamerman, 1956]. In contrast, the FBS-grown spheroids contained elongated, fibroblast-like cells and no detectable metachromatically stained extracellular matrix (Fig. 3A). Also, the Nutridoma-grown spheroids were organized into two morphologies in distinct cellular compartments, whereas cellular organization was random in FBS-grown spheroids (Fig. 3). In addition, immunocytochemistry of spheroids grown in Nutridoma revealed significantly greater type II and III collagen at days 7 and 10 than spheroids grown in FBS (not shown). Untreated 10T1/2 micromasses, at day 7, did not form spheroids (Fig. 2), did not stain with Azure at pH 1, and displayed no histomorphological similarities with the BP-treated spheroids (not shown). Since Nutridoma significantly increased several parameters of chondrogenesis when compared to FBS, subsequent experiments were performed in culture medium containing Nutridoma.

Dose-Dependent Chondrogenic Response to BP

In the first set of studies, we verified that BP was required for spheroid formation and that Nutridoma promotes more extensive chondrogenesis than FBS (Figs. 2, 3). To determine the effect of BP concentration on spheroid formation, concentrations of BP ranging from

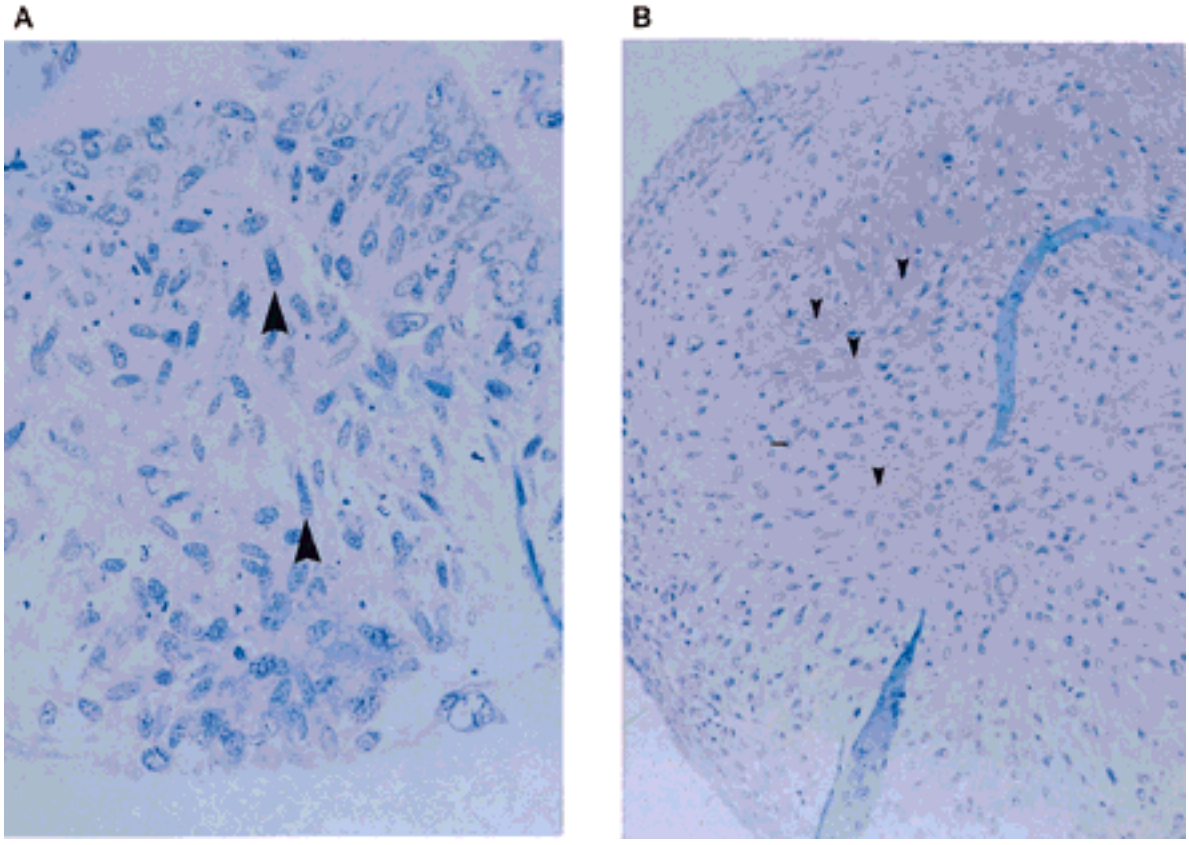

Fig. 3. Extent of chondrogenesis in two differentiation conditions. 10T1/2 micromass cultures were induced to differentiate with 0.5 μg/ml BP in the presence of FBS (**A**) or in 1% Nutridoma (**B**). After 7 days, the resulting spheroids were sectioned and stained with Azure at pH 1. Note the elongated cells (**A,** *arrowheads*) and the extensive areas of diffuse metachromatic stain (**B,** pink areas as indicated by *arrowheads*). A: ×200. B: ×100.

.02–12.5 μg/ml were tested (Table I). Spheroid formation occurred most quickly with BP concentrations of 0.1–2.5 μg/ml. BP concentrations of 12.5 μg/ml resulted in a significantly larger spheroid, although appearance was delayed, whereas 0.02 μg/ml BP concentration resulted in no spheroid formation (Table I).

To determine the relationship between BP concentration and the extent of chondrogenesis in the spheroid, we grew 10T1/2 micromasses in the presence of 0.1, 0.5, 2.5, and 12.5 μg/ml BP in Nutridoma media. The resulting spheroids were analyzed by histology and immunocytochemistry at days 7 and 10. We noted a direct relationship between the amount of BP and the amount of metachromatically stained matrix (Table I). The higher the concentration of BP that was added to the culture, the greater the amount of intracellular and extracellular metachromatically stained material produced. A similar correlation between amounts of type I, II, and III collagens and BP concentration was also observed (not shown).

Chondrogenic Maturation Is Observed Over 28 Days

To demonstrate the progression of chondrogenesis over time, we induced 10T1/2 micromasses to differentiate with 0.5 μg/ml BP, and the resulting spheroids were harvested at day 2 and at weekly intervals through day 28 and stained with Azure at pH 1 (Figs. 3B, 4). At day 2, the spheroid contained densely packed, randomly organized cells, and no staining was detected at pH 1 (Fig. 4A). However, by day 7, extensive, diffusely stained metachromatic matrix was observed at pH 1 (Fig. 3B). On day 14, staining at pH 1 revealed granular extracellular metachromatic staining (not shown). By day 21, metachromatic staining became more intense, forming a high density matrix surrounding individual rounded cells (Fig. 4B). This was the first detected appearance of individual chondrocyte morphology. By day 28, the density and amount of metachromatic staining had increased, and a distinct territorial matrix had formed (Fig. 4C).

TABLE I. Dose Dependent Chondrogenic Response to BP*

BP (μg/ml)	0.02	0.1	0.5	2.5	12.5
Spheroid formation	N	Y	Y	Y	Y^d
Metachromasia	N/D	+	++	+++	++++

*10T1/2 micromass cultures were induced to differentiate with BP concentrations ranging from 0.02 μg/ml to 12.5 μg/ml BP. Micromass cultures were grown in FBS and analyzed for spheroid formation or in 1% Nutridoma and stained with Azure at pH 1 for metachromasia content. 'N,' 'Y,' and 'Y^d,' indicate no spheroid formation after 11 days, spheroid formation after three days, or delayed spheroid formation, occurring in days 7 through 11, respectively. '+' indicates that detectable metachromasia was observed and increasing '+' indicates observed increased intensity and quantity of the stain.

To further enhance chondrogenesis, we added 50 μM ascorbic acid and 10 mM β-glycerophosphate to 10T1/2 spheroids at day 18, and the media was replaced with new media containing ascorbic acid and β-glycerophosphate on day 25. These conditions promoted formation of a more concentrated metachromatic matrix and individual, hypertrophic chondrocytes surrounded by a territorial matrix at day 28 (Fig. 4D).

Throughout the time course of this experiment, the spheroid was composed of two distinct compartments as visualized by Azure stain (Fig. 4C). The outer rim of the spheroid, which is in contact with the media, consisted of closely packed arrays of cells that morphologically resembled a perichondrium. This encompassed the inner, chondrogenic core of the spheroid. The majority, if not all, of the cells in the spheroid appeared to be surrounded by metachromatically stained material, which is characteristic of cartilage extracellular matrix. Significantly, no characteristic cellular morphologies of undifferentiated fibroblast or differentiated muscle, fat, or bone cells were observed throughout the time course of this experiment (Fig. 4).

Immunocytochemistry and Electron Microscopy Revealed Additional Markers Consistent With Chondrogenesis

To unequivocally document that cells had undergone a chondrogenic fate in culture, 10T1/2 micromass cultures were treated with 0.5 μg/ml BP and harvested at weekly intervals over 28 days, and the spheroids were assayed for expression of cartilage-specific proteins by immunocytochemistry. Immunocytochemical staining revealed the appearance of cartilagenous components during a similar time frame as appearance of metachromatic staining (Figs. 4, 5). At day 2 after BP exposure, collagens were faintly or not detected [Atkinson et al., 1996].

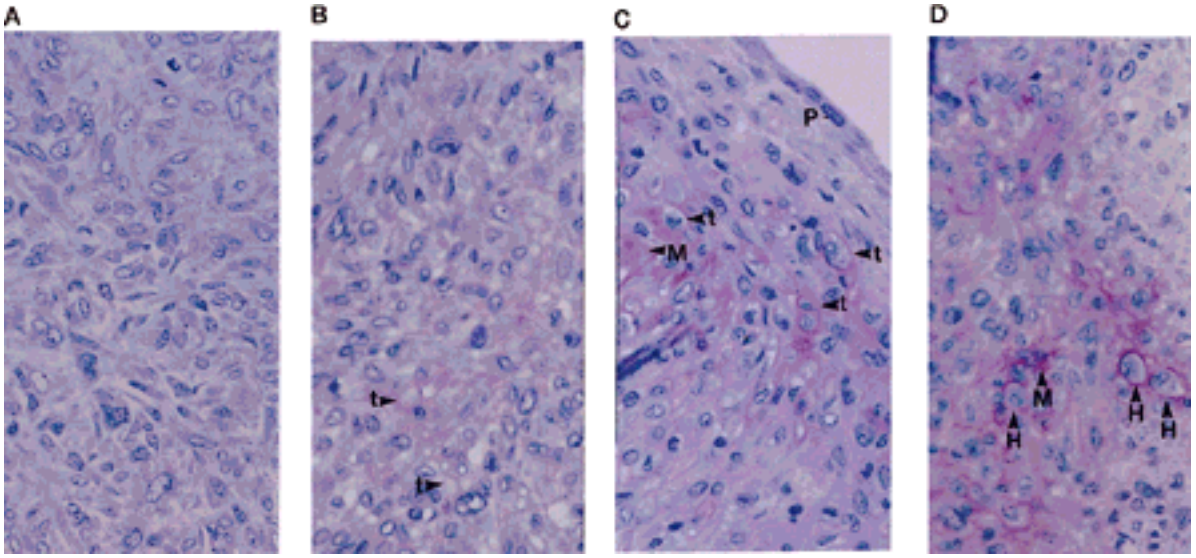

Fig. 4. Chondrogenic maturation over 28 days was enhanced by ascorbic acid and β-glycerophosphate. Micromass cultures were grown in Nutridoma media containing 0.5 µg/ml BP, and spheroids were harvested at day 2, 21, and 28 (**A,B,C,** respectively). Note the perichondrium-like region (P) that encompasses a diffuse (B) and distinct (C) territorial matrix (t). D: On day 18 and day 25, 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate were added prior to spheroid harvest at day 28. Note a more well-defined territorial matrix (T) and hypertrophic chondrocytes (H). Also note the increase in intensity and range of metachromasia in C and D compared to B. ×200.

However, by days 7 and 10, large amounts of type II collagen were detected, indicative of an early cartilagenous stage [Atkinson et al., 1996]. By day 21, the quantity of type II collagen had increased, and the presence of link protein was observed in a lacunar distribution (Fig. 5). In addition, osteocalcin, which is correlated with the appearance of hypertrophic chondrocytes [Neugebauer et al., 1995], is observed at day 14, and the quantities increase at day 21 (Fig. 5). The presence of link protein, a component of aggregating proteoglycan, type II collagen, the principal protein of cartilage [Linsenmayer et al., 1973; Solursh et al., 1981; Swalla and Solursh, 1984], and osteocalcin provides strong evidence for the presence of cartilage.

The presence of proteins observed in both cartilagenous and in noncartilagenous tissues was also determined. These were classified as nonspecific cartilage markers. At days 7 and 14, types I and III collagen were detected, and, by day 21, the quantities of each of these proteins had increased (Fig. 5). Bone sialoprotein (BSP) and osteopontin (OP), which have been observed in hypertrophic chondrocytes and in bone, were not detected at days 14 and 21 or throughout the 28 day time course (BSP and OP in Fig. 5). Sections from day 14 and 21 tissue that was incubated with nonimmune serum revealed no detectable stain (C in Fig. 5).

Because significant extracellular matrix was produced with 12.5 µg/ml BP treatment, electron microscopy (EM) was performed on 10T1/2 micromass cultures treated with this concentration of BP. Seven days after BP addition, EM analysis of the spheroid revealed rounded cells separated by abundant extracellular matrix (Fig. 6A,B). The cells contained elongated processes that resembled psuedopodia (Fig. 6A), which are characteristic of cartilagenous cells [Stockwell, 1979]. The cells also contained distended endoplasmic reticulum and secretory vescicles (Fig. 6A). Additionally, the extracellular matrix was composed of short, disorganized arrays of collagen fibrils typical of true cartilage matrix (Fig. 6B,C). We also observed the characteristic collagen cross-banding pattern on these fibrils (not shown). Characteristic proteoglycan structures and a structure resembling a calcifying vescicle were also observed (Fig. 6C). After only a week in culture, in the absence of ascorbic acid and β-glycerophosphate, significant cartilage morphological features were observed.

Spheroids Contain No Adipocyte Morphology or Signs of Overt Mineralization

To determine whether adipocytes were present in the spheroids, sections from spheroids were stained with Oil Red O, which detects

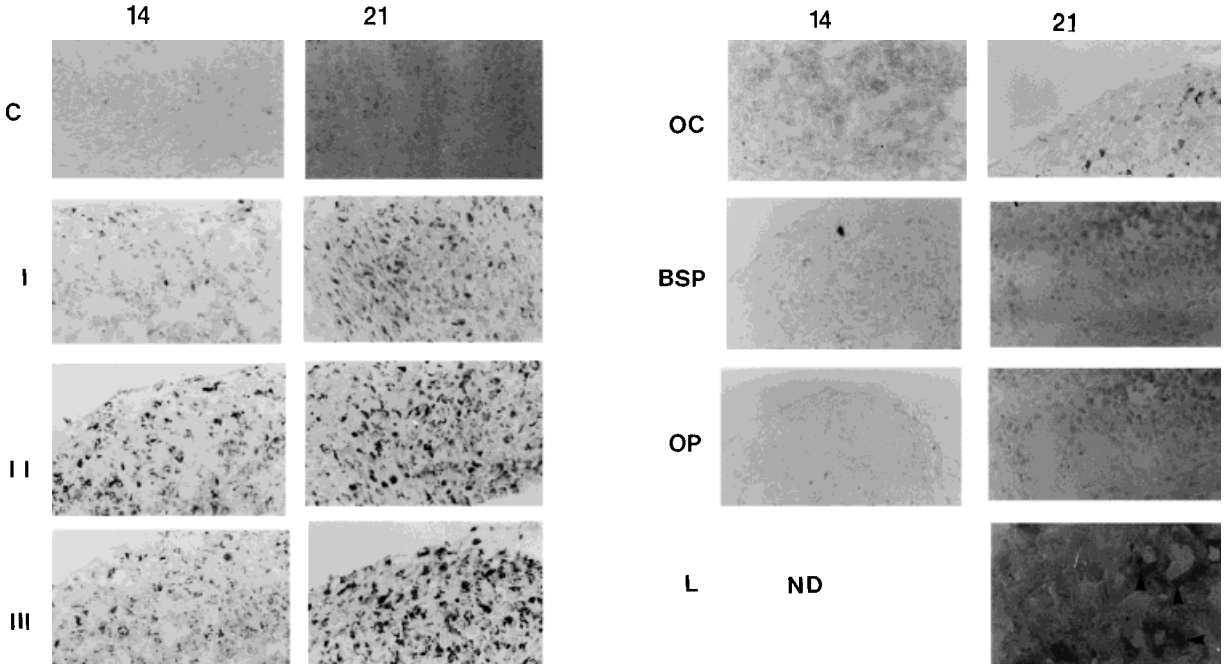

Fig. 5. Immunocytochemical analysis of chondrogenesis induced with BP. 10T1/2 micromass cultures were grown in 1% Nutridoma media supplemented with 0.5 μg/ml BP and harvested at day 14 (14) and day 21 (21). Serial sections from the resulting spheroids were stained with nonimmune serum (C), antibodies to type I, II, or III collagens (I, II, III, respectively), osteocalcin (OC), bone sialoprotein (BSP), osteopontin (OP), or link (L; *arrowheads*). Only the link section was counterstained with hematoxylin to visualize nuclei. Magnification was ×200 for all stains except link, which was ×600.

neutral lipid [Lillie and Fulmer, 1976]. Neutral lipid in chondrocytes was first described in 1849 [Leidy, 1849] and is a normal component of adult cartilage [Irving and Wuthier, 1968; Montagna, 1949; Stockwell, 1967]. Oil Red O was detected at day 7 in low quantities which increased at weekly intervals through day 28 (Fig. 7). The positively staining vescicles were small (1–10 μm) and distributed randomly throughout the cell, as expected for cartilagenous cells (Fig. 7). Note that the large, organized vesicles characteristic of adipocytes are not observed. To determine whether the spheroids had undergone overt mineralization, we performed von Kossa staining. No positive stain was observed over the time course of this experiment (not shown).

DISCUSSION

In this report, we demonstrate in vitro differentiation of 10T1/2 mesenchymal cells to the cartilage lineage with high efficiency and specificity and define the optimal conditions for this process to occur. A key feature of this optimization appears to be the use of a mixture of bone-derived proteins, BP, that have been purified on the basis of an in vivo osteoinduction assay. While BP treatment of 10T1/2 cells in monolayer had no detectable morphological effect, BP treatment of 10T1/2 cells in micromass cultures consistently resulted in the formation of a three-dimensional structure, termed a spheroid (Fig. 2) [Denker et al., 1995]. The spheroid consists of a distinct perichondrium-like structure that encompasses the region undergoing overt chondrogenesis (Fig. 4). Nearly all of the cells within the spheroid appear to be undergoing chondrogenesis; no evidence of bone, muscle, or fat cells is observed (Figs. 4, 6, 7). Furthermore, chondrogenesis in vitro proceeds through discrete stages, ultimately to the formation of individual chondrocytes, that appears to faithfully recapitulate the differentiation steps observed in vivo (Table II). These data indicate that the BP fraction of proteins contains not only factors that induce appropriate cartilage differentiation but also those that inhibit stem cell differentiation into the other mesengenic lineages.

Previous studies, which utilized 10T1/2 confluent, monolayer cultures grown in serum containing a single, osteoinductive factor (BMP-2 or BMP-4), achieved low rates of differentiation (0.2% for BMP-2) and differentiation to three mesengenic lineages [Ahrens et al., 1993; Kata-

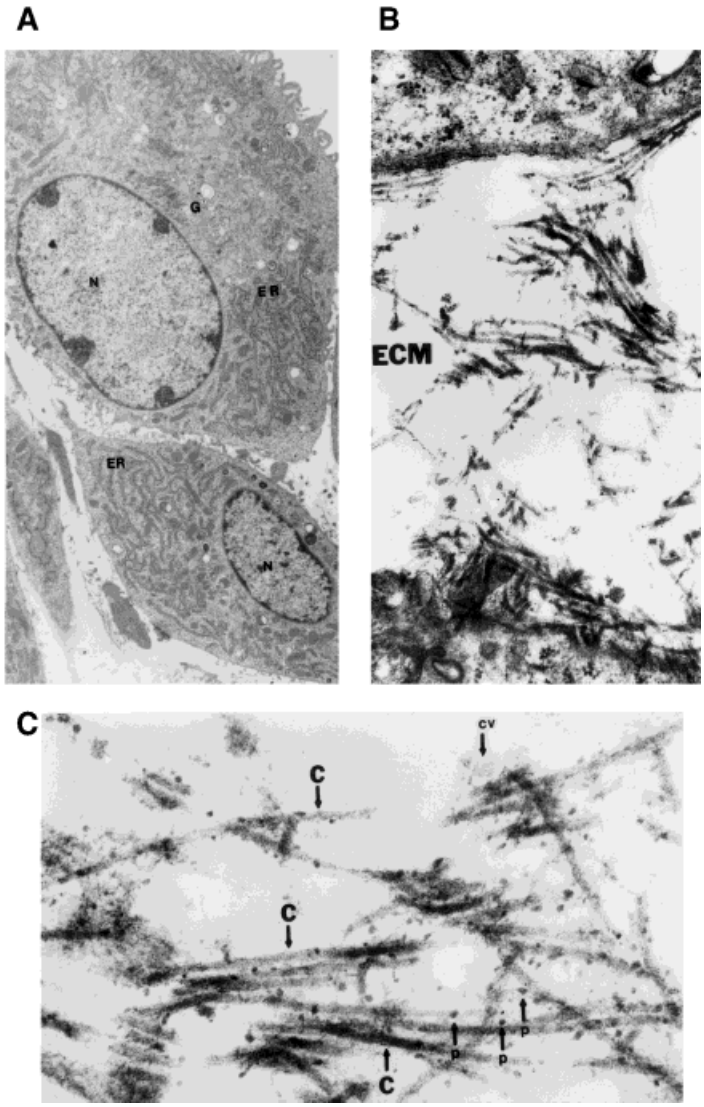

Fig. 6. Cartilage morphological characteristics detected by electron microscopy. 10T1/2 micromass cultures were induced to differentiate with 12.5 μg/ml BP and 1% Nutridoma. The resulting spheroid was harvested at day 7, and electron microscopy was performed at three levels of magnification. Photomicrographs from the lowest magnification (**A**) showed rounded cell morphology, pseudopodia, and distended endoplasmic reticulum (ER). Additional cellular organelles identified were the nucleus (N) and the Golgi apparatus (G). The intermediate magnification photomicrograph (**B**) shows cells separated by extracellular matrix (ECM) containing arrays of collagen fibrils. The highest magnification photomicrograph (**C**) reveals the components of the extracellular matrix including the short, disorganized arrays of collagen fibrils (C), proteoglycan (p), and a structure resembling a calcifying vescicle (cv). Magnification was 1000×, 7000×, and 30000× for A, B, and C, respectively.

giri et al., 1990; Wang et al., 1993]. In contrast, BP treatment of monolayer cultures resulted in no differentiation to adipocytes (Fig. 1). The BP mix may contain factors that preclude differentiation to the adipogenic lineage, whereas individual BMP-2 and BMP-4 proteins may lack these inhibitory functions. In addition, cells within a monolayer may be exposed to heterogenous microenvironments of differential cell densities, cell contacts, oxygen tension, and nutrient availability, which may affect the ultimate developmental fates. In contrast, the micromass cultures may provide a more highly organized environment with respect to these features. Specifically, cell-cell contacts occur in three dimensions for essentially each cell in the micromass; gradients of nutrients and oxygen tension are established; and such gradients may provide inside/outside positional cues to the cells [Freshney, 1994]. Although micromass con-

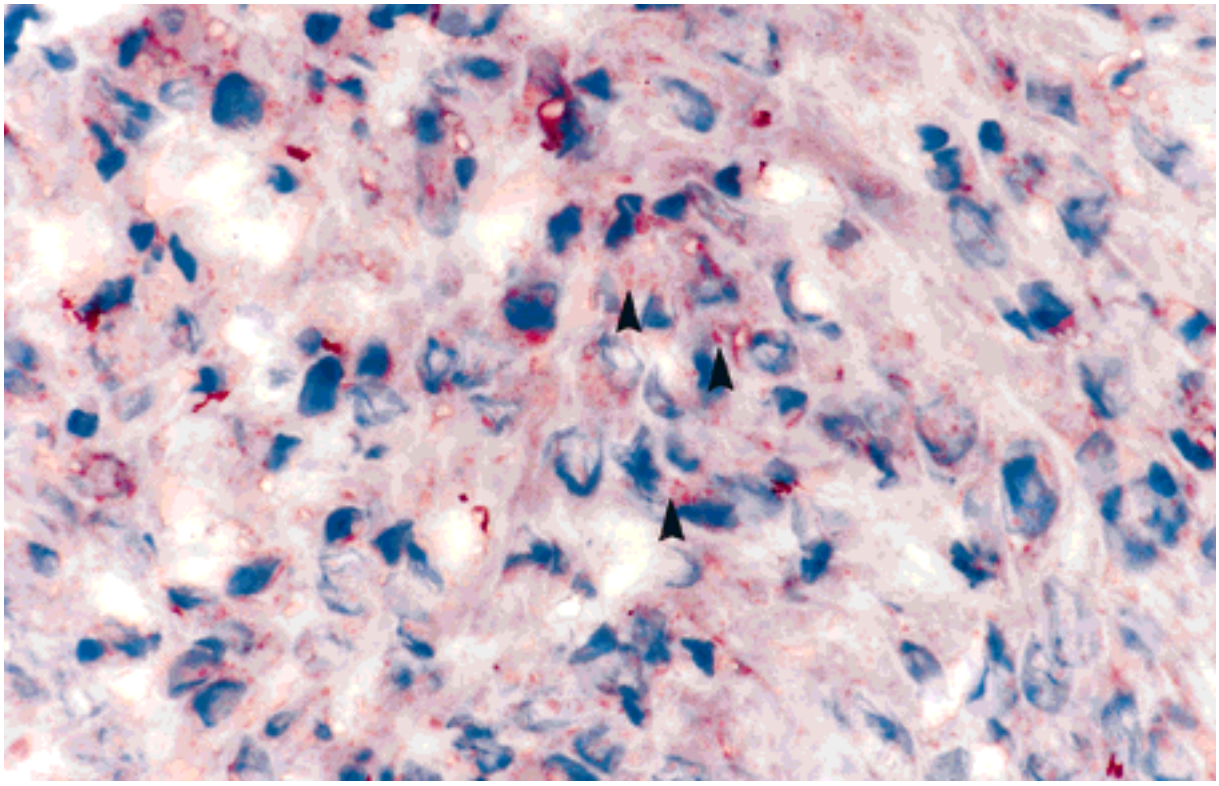

Fig. 7. Oil Red O staining detects small lipid vescicles. 10T1/2 micromass cultures were induced to differentiate with 0.5 µg/ml BP and 1% Nutridoma. The resulting spheroid was harvested at day 28 and stained with Oil Red O. *Arrowheads* indicate small lipid vescicles (1–10 µm) characteristic of cartilage but not adipocytes. ×600.

ditions alone are insufficient to drive cartilage development, they are clearly necessary for BP chondroinduction.

During normal cartilage ontogeny, multiple BMPs act on mesenchymal stem cells to induce discrete differentiation steps, beginning with a mesenchymal condensation stage. To faithfully recapitulate cartilage ontogeny, a combination of purified osteoinductive bone proteins was used to mimic the combinations of BMPs that are important for normal cartilage development and thus induce the condensation stage [Rosen, 1996]. Micromass cultures have been shown to be crucial for both lineage commitment to chondrogenesis and lineage repression to related mesengenic pathways in limb bud explants [Ahrens et al., 1977; Caplan, 1970; Ede, 1983; Hall, 1983; Osdoby and Caplan, 1979]. Micromass culture is also crucial to spheroid formation, which has been described previously [Denker et al., 1995]. Cell surface adhesion molecules (cadherin, activin, fibronectin, cellular adhesion molecule(s), etc.) and/or reduced oxygen tension may mediate signal transduction events that favor chondrogenesis. Moreover, during embryonic cartilage development, multiple BMPs are expressed. BMP-2 and -5 are expressed in the condensing mesenchyme, and BMP-2, -4, and -6 have been localized to areas undergoing skeleton formation [Kingsley, 1994; Lyons et al., 1989, 1990]. Also, individual BMPs can form heterodimers, and these heterodimers may be more active [Aono et al., 1995] and/or provide a combinatorial array that may direct the complexity involved in normal skeleton formation more than individual or homodimeric BMPs. Furthermore, naturally occuring mutations in the BMP-5 gene do not result in a cartilage- or bone-deficient mouse but rather in short ears [Kingsley, 1994]. This effect is thought to be due to the redundancy of BMP effectors, since the distal portion of the ear is one site where BMP-5 is soley expressed [King et al., 1996]. Given that the purification scheme used to isolate BMPs was similar to the purification of the BP fraction and that BP is essentially an earlier step, preliminary data suggest that BP is composed of

TABLE II. The Chondrogenic Lineage and Temporal Appearance of Cartilage Markers*

Stage of Differentiation	Characterized by:	Day observed
Mesenchymal Stem Cell		
↓		
Committed Chondroprogenitor	type I collagen	7 → 28
	CSPG-M	not tested
	Metachromasia	7 → 28
↓		
Chondroblast	type II collagen	7 → 28
↓	CSPG-H	not tested
Chondrocyte I	Link protein	21
↓	Territorial matrix	21 → 28
Hypertrophic Chondrocyte	type X collagen	not tested
	Increased cell size	28
↓		
Calcifying Hypertrophic Chondrocyte	type I collagen	7 → 28
	Osteocalcin	21 → 28
	Bone sialoprotein	not observed
	Osteopontin	not observed
	von Kossa	not observed

*Stages of chondrogenic maturation from a mesenchymal stem cell to the calcifying hypertrophic chondrocyte are shown, as previously described [Bruder et al., 1994]. Each stage was defined by biosynthesis of specific proteins which were detected immunocytochemically [Bruder et al., 1994; Solursh et al., 1981; Swalla and Solursh, 1984]. The day that each particular marker was observed in culture is denoted. The detection of metachromasia and territorial matrix as markers for the Committed Chondroprogenitor and Chondrocyte I stages, respectively, were added by the authors. CSPG-H is chondroitin sulfate proteoglycan (aggrecan).

combinations of BMP homo- and heterodimers, as observed previously [Wozney, 1993]. Thus, BP may more efficiently mimic in vivo chondrogenesis than individual growth factors (e.g., BMP-2, BMP-4, TGFβ-1). Also, 10T1/2 cells grown as micromass cultures responded to TGFβ-1 by producing a spheroid, although differentiation proceeded only to a very early stage in chondrogenesis (precartilagenous condensation stage) in which no chondrocyte morphology was observed [Denker et al., 1995]. The unique combination of conditions reported in this manuscript provides the optimal conditions for differentiation to more mature chondrogenic stages.

The specific combination of media components that we have identified (i.e., a serum substitute containing insulin and transferrin, ascorbic acid, β-glycerophosphate, and BP) was responsible for the more efficient differentiation to mature cartilage. Low serum promotes cell growth arrest and is correlated with progression of both myogenesis and osteogenesis [Halevy et al., 1995; Stein and Lian, 1993]. Since differentiation is inversely related to proliferation [Stein and Lian, 1993], culture conditions must be carefully established to maximize chondrogenesis over mitogenesis. Chondrogenesis was inhibited in cultures containing both BP and serum, in which excessive growth may occur (Fig. 3). Furthermore, micromasses cultured in the presence of serum substitute appeared to die over time, whereas the addition of BP promoted chondrogenesis (Fig. 2). Ascorbic acid has been shown to stimulate both collagen biosynthesis and in vitro differentiation of chondrocytes; the formation of collagenous extracellular matrix has been proposed to promote further differentiation along the cartilage lineage [Franceschi, 1992; Wu et al., 1989]. Furthermore, insulin, transferrin, ascorbic acid, and β-glycerophosphate have been shown to promote chondrogenesis and osteogenesis [Bohme et al., 1992; Cancedda et al., 1992; Franceschi, 1992; Goldring et al., 1994; Kato et al., 1980; Owen et al., 1990; Quarto et al., 1992; Stevens et al., 1981; Takigawa et al., 1991]. In this report, the addition of ascorbic acid (50 μg/ml) and β-glycerophosphate (10 mM) enhanced BP-dependent proteoglycan production, chondrocyte morphology, and appearance of lacunae (Fig. 4D compared to Fig. 4C).

The chondrogenic phenotype of differentiated 10T1/2 cells was verified by several criteria, including electron microscopy (Fig. 6). Electron photomicrographs of the in vitro cultures share similar characteristics with adult articular cartilage and 2-week-old epiphesial cartilage [Stockwell, 1979]. These similarities include rounded cell morphology, pseudopodia, distended endoplasmic reticulum, secretory vesicles, cells separated by extracellular matrix, short, disorganized collagen fibrils, and proteoglycans as early as 7 days after BP addition (Fig. 6).

The appearance of histological and immunocytochemical markers over time in culture revealed similarities with chondrogenic stages observed in vivo, as described previously [Bruder et al., 1994] (Table II). Markers that characterize the early committed chondroprogenitor and the chondroblast stages of chondrogenesis (type I and type II collagen, respectively), were observed as early as day 7, and the quantity increased over time (Fig. 5). Markers

for the intermediate chondrocyte I stage (link protein, territorial matrix), were observed at an intermediate time point (day 21). Type III collagen was also expressed at low levels at day 2 and increasing levels through day 28. Type III collagen is a nonspecific, ubiquitous marker that has been shown to be expressed in adipocytes and nondifferentiated mesenchymal cells. In addition, type I, II, and III collagens have been reported to be coexpressed in newly synthesized cartilage [Sandberg et al., 1993]. Increased cell size is a phenotype correlated with the hypertrophic chondrocyte stage and was faintly observed at day 21 and clearly observed at day 28 (Fig. 4). Further differentiation to the calcifying hypertrophic chondrocyte stage involves mineralization. Markers correlated with overt mineralization, such as von Kossa stain, and bone sialoprotein and osteopontin were not observed. We conclude that differentiation proceeds to morphologically hypertrophic chondrocytes and that overt mineralization is precluded.

The transformation from embryonic mesenchyme to cartilage in vivo begins when undifferentiated mesenchymal cells undergo active cell-cell interactions, migrate to a specific location in the embryo, condense, and secrete large amounts of cartilagenous extracellular matrix [Ede, 1983; Hall, 1978; Rosen and Thies, 1992]. Fibroblast cells form around each cartilage nodule, creating a boundary between the developing cartilage and the surrounding tissue. The final maturation of cartilage involves hypertrophy of cartilage cells followed by mineralization of the matrix. We observed similarities between embryonic and in vitro differentiation to cartilage. BP treatment of mesenchymal 10T1/2 cells, in high density micromass cultures, leads to a uniform separation event that promotes condensation of the inner mass of cells away from a surrounding ring of undifferentiated fibroblasts. The three-dimensional spheroid resulting from the condensation contains two morphologically distinct components in cross-section: an interior region undergoing chondrogenesis that is encompassed by a perichondrium-like structure (Fig. 4). Furthermore, cross-sections of the spheroid resemble both the cross-section of a developing forelimb of a 13.5 days postcoitum mouse embryo [Rosen and Thies, 1992] and the transverse section of a stage 31 chick forelimb digit cartilage [Gould et al., 1974]. We conclude that BP and the reported culture conditions induce pattern formation during chondrogenic differentiation, from murine mesenchymal stem cells, that is similar to chondrogenesis observed during embryonic limb formation.

This in vitro model for chondrogenesis provides a defined system to study factors that regulate mesengenic differentiation, including those that regulate exclusion to related mesengenic pathways, commitment to the cartilage lineage, and maturation along the chondrogenic pathway. As such, it provides a convenient alternative to the limb bud explant cultures in current use. In addition, it provides the framework for future autologous cell therapy approaches for cartilage repair. Pluripotential stem cells could be amplified and induced to differentiate to the cartilage lineage ex vivo, prior to reintroduction to the patient. By manipulating conditions in vitro, this approach may recapitulate cartilage ontogeny and provide a cartilage substitute that closely resembles, in mechanical strength and structure, endogenous cartilage.

ACKNOWLEDGMENTS

We thank A. Denker for helpful discussions and sharing results prior to publication, P. Ruegg and M. Ryan for histology and immunocytochemistry technical assistance, and A. Jones for the electron microscopy work. In addition, we thank Genetics Institute for human recombinant BMP-2. We thank A. Bradford, S. Diamond, and D. Gordon for critical reading of this manuscript. This work was supported by a Colorado Institute for Research in Biotechnology grant and matching support from Intermedics Orthopedics, Inc. Additionally, B.L.A. received partial support from the Lucille P. Markey Charitable Trust. The osteopontin (MPIIIBIO), sialoprotein (WVIDI), and link protein (9/30/8.A.4) antibodies were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine (Baltimore, MD), and the Department of Biological Sciences, University of Iowa (Iowa City, IA), under contract N01-HD-6-2925 from the NICHD.

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