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BMP-2 Induction and TGF-β1 Modulation of Rat Periosteal Cell Chondrogenesis

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Abstract Periosteum contains osteochondral progenitor cells that can differentiate into osteoblasts and chondrocytes during normal bone growth and fracture healing. TGF-β1 and BMP-2 have been implicated in the regulation of the chondrogenic differentiation of these cells, but their roles are not fully defined. This study was undertaken to investigate the chondrogenic effects of TGF-β1 and BMP-2 on rat periosteum-derived cells during in vitro chondrogenesis in a three-dimensional aggregate culture. RT-PCR analyses for gene expression of cartilage-specific matrix proteins revealed that treatment with BMP-2 alone and combined treatment with TGF-β1 and BMP-2 induced time-dependent mRNA expression of aggrecan core protein and type II collagen. At later times in culture, the aggregates treated with BMP-2 exhibited expression of type X collagen and osteocalcin mRNA, which are markers of chondrocyte hypertrophy. Aggregates incubated with both TGF-β1 and BMP-2 showed no such expression. Treatment with TGF-β1 alone did not lead to the expression of type II or X collagen mRNA, indicating that this factor itself did not independently induce chondrogenesis in rat periosteal cells. These data were consistent with histological and immunohistochemical results. After 14 days in culture, BMP-2-treated aggregates consisted of many hypertrophic chondrocytes within a metachromatic matrix, which was immunoreactive with anti-type II and type X collagen antibodies. In contrast, at 14 days, TGF-β1+BMP-2-treated aggregates did not contain any morphologically identifiable hypertrophic chondrocytes and their abundant extracellular matrix was not immunoreactive to the anti-type X collagen antibody. Expression of BMPR-IA, TGF-β RI, and TGF-β RII receptors was detected at all times in each culture condition, indicating that the distinct responses of aggregates to BMP-2, TGF-β1 and TGF-β1+BMP-2 were not due to overt differences in receptor expression. Collectively, our results suggest that BMP-2 induces neochondrogenesis of rat periosteum-derived cells, and that TGF-β1 modulates the terminal differentiation in BMP-2 induced chondrogenesis. J. Cell. Biochem. 81:284–294, 2001.

Key words: periosteum; BMP-2; TGF-β1; hypertrophy; chondrogenesis

Periosteum plays a crucial role in bone development and fracture healing. In normal bone growth, periosteum-derived cells differentiate directly into osteoblasts [Taylor, 1992]. In fracture healing, periosteum-derived cells differentiate into chondrocytes and osteoblasts that participate in endochondral ossification and intramembranous ossification [Simmons, 1985; Bolander, 1992]. These studies indicate that the periosteum contains mesenchymal progenitor cells, which are capable of differentiating into either osteoblasts or chondrocytes depending on a variety of local factors including mechanical and biochemical stimuli.

TGF-β1 causes induction of localized chondrogenesis when injected subperiosteally [Joyce et al., 1990], facilitates in vitro chondrogeneic differentiation of chick periosteal cells, rabbit periosteal explants [Iwasaki et al., 1993; O'Driscoll et al., 1994] and induces mRNA expression of type II collagen in monolayer cult-

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ures of bovine periosteal cells [Izumi et al., 1992]. In addition, TGF-β1 is a potent promoter of the chondrogenic differentiation of embryonic limb mesenchymal cells in vitro [Kulyk et al., 1989; Schofield and Wolpert, 1990] and stimulates fibroblastic mesenchymal-like cells isolated from fetal rat muscle explants to synthesize matrix proteins characteristic of cartilage [Syedin et al., 1985]. However, the effects of TGF-β1 on terminal differentiation of chondrocytes are less clear. TGF-β1 induced early mRNA expression of type X collagen, a marker of hypertrophy, in chick periosteal cell culture [Iwasaki et al., 1993], but it delayed the hypertrophic differentiation in epiphyseal and rib chondrocyte cultures [Kato et al., 1988; Ballock et al., 1993].

Bone morphogenetic protein-2 (BMP-2) is present in the pre-cartilaginous mesenchyme of embryonic tissue and in undifferentiated periosteal cells of early fracture callus of postnatal bone [Bostrom et al., 1995]. BMP-2 stimulates chondrogenic differentiation in cultures of a murine mesenchymal pluripotential cell line C3H10T1/2 and in micromass culture of chick limb mesenchymal cells when added exogenously [Wang et al., 1993; Roark and Greer, 1994]. However, the chondrogenic effects of BMP-2 on periosteal cells derived from postnatal animals have not been investigated extensively. To our knowledge, there is only one report, which indicated that BMP-2 did not affect chondrogenic differentiation in high density culture of chick periosteum-derived cells [Iwasaki et al., 1994].

We have successfully developed a three-dimensional aggregate culture system in which bone marrow-derived mesenchymal progenitor cells terminally differentiate into hypertrophic chondrocytes [Johnstone et al., 1998; Yoo et al., 1998]. This model maintains cells as an aggregate, thereby more closely approximating the three-dimensional environment of developing cartilage in vivo. Moreover, this system allows us to study the effects of growth factors on progression of chondrogenesis from progenitor cells, since serum is not added to the cultures and all components of culture medium are defined. The aim of this study was to examine the effects of BMP-2 and TGF-β1 on the chondrogenic differentiation of rat periosteum-derived cells in this culture system. The results of gene expression analysis and immunohistochemical localization of cartilage-specific matrix proteins indicated that BMP-2, but not TGF-β1, stimulates neochondrogenesis of rat periosteum-derived cells, and that TGF-β1 modulated terminal differentiation in BMP-2 induced chondrogenesis.

MATERIALS AND METHODS

Isolation and Expansion of Rat Periosteal Cells

Periosteal explants were harvested by careful dissection from the medial distal femurs (2 mm distal to the distal femoral epiphyseal growth plate) of 2-month-old male Fisher 344 rats (Charles River Laboratory, Wilmington, MA) and placed on a 100-mm culture dish (Becton-Dickinson Labware, Franklin Lakes, IN) with the cambium side (bone surface) of periosteum against the dish. The explants were cultured in Dulbecco's modified Eagle's medium–low glucose (DMEM-LG) (Gibco BRL, Gaithersburg, MD) containing 10% of a selected lot [Lennon et al., 1996] of fetal bovine serum (FBS) (Gemini Bio-products, Inc., Calabasa, CA) and antibiotic–antimycotic solution (penicillin, streptomycin and fungisone, Gibco BRL) at 37°C in 95% humidified air and 5% CO_2 for a week. Cells that migrated from the periosteal explants were released by exposure to 0.25% trypsin/1 mM EDTA (Gibco BRL) for 5 min at 37°C, followed by the addition of one-half volume of calf serum (Hyclone Laboratories, Logan, UT) to stop the reaction. The released cells were then centrifuged, resuspended in complete medium (DMEM-LG supplemented with antibiotic–antimycotic solution and 10% FBS), and seeded at 7×10^4 cells/100-mm dish. At 10 days of culture, before reaching confluence, the expanded periosteal cells were trypsinized, recovered by centrifugation at 1000 rpm for 5 min, and used for experiments described in the following sections. At the same time, two dishes were not trypsinized and cultured cells were stained for alkaline phosphatase (ALP).

Cytochemical Analysis for ALP

The medium was removed from monolayer-expanded periosteal cells and they were fixed in citrate-buffered acetone for 1 min. After rinsing with distilled water, ALP activity was detected with a commercial kit (Sigma, St. Louis, MO) following the supplier's instructions.

Aggregate Culture of Periosteal Cells

Periosteal cells, isolated from explants and monolayer-expanded, were cultured as three-dimensional cell aggregates as previously described [Johnstone et al., 1998; Yoo et al., 1998]. Briefly, after washing with a defined medium consisting of DMEM-high glucose with 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 μg/ml selenious acid, 1.25 μg/ml bovine serum albumin (BSA), 5.35 μg/ml linoleic acid, 1 mM pyruvate (Gibco BRL), and 50 μg/ml ascorbate 2-phosphate (Wako, Osaka, Japan), harvested periosteal cells were centrifuged at 1000 rpm for 5 min and resuspended in the defined medium at a final concentration of 2×10^5 cells/ml. Aliquots (1 ml) were added to 15 ml conical polypropylene centrifuge tubes (Becton-Dickinson Labware) and the cells pelleted by centrifugation at 1000 rpm for 5 min. The medium in each tube was replaced with 0.5 ml defined medium containing 10 ng/ml recombinant human rhTGF-β1 (R&D Systems, Minneapolis, MN), 25 ng/ml BMP-2 (a generous gift from Genetics Institute, Cambridge, MA), or both. These factors were added with each change of the culture medium. Control cultures were maintained without adding TGF-β1 or BMP-2. The pellets were then incubated at 37°C in 95% humidified air and 5% CO_2 . Within 24 h, the cells had formed a free-floating aggregate. Culture medium was changed every other day. The aggregates were harvested on days 3, 7, 11 and 14. Four independent cultures were performed to confirm the reproducibility of the results.

Total RNA Extraction, cDNA Synthesis, and RT-PCR Analysis

Total RNA was extracted from 10 pellets on days 3 and 7, and 20 pellets on days 11 and 14 with a commercial kit (Qiagen Inc., Chatsworth, CA) following the manufacturer's instruction. The purity and amount of isolated RNA were assessed by spectrophotometric measurement at 260 and 280 nm. Total RNA (1 μg) was reverse transcribed to cDNA at 42°C for 50 min in a volume of 20 μl containing the following reagents: 0.5 mM dNTP mix; 10 mM dithiothreitol; 0.5 μg oligo$(dT)_{12-18}$, 1 × first strand buffer (5 × = 250 mM Tris, pH 8.3, 375 mM KCl and 15 mM $MgCl_2$) and 20 U of Superscript II (RNase H-free reverse transcriptase) (all from Gibco BRL). After terminating the reaction at 70°C for 15 min, 1 U of RNase H (Gibco BRL) was added to the reaction mixture, followed by incubation at 37°C for 10 min to remove the RNA.

Aliquots of the cDNA were diluted 1:500 and then amplified in 50 μl of a PCR reaction mixture which contained 20 pmol primer sets, 1 × PCR buffer (10 × 200 mM Tris, pH 8.4, and 500 mM KCl), 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, and 2.5 U of *Taq* DNA polymerase (all from Gibco BRL). Primers for aggrecan core protein (sense: 5′-TAGAGAAGAAGAGGGGTTAGG-3′ and antisense: 5′-AGCAGTAGGAGCCAGGGTTAT-3′ from bases 4810 to 5131), α2(I) collagen (sense: 5′-GGTGGTTATGACTTTGGTTAC-3′ and antisense: 5′-CAGGCGTGATGGCTTATTTGT-3′ from bases 3785 to 4466), type II collagen (sense: 5′-GAAGCACATCTGGTTTGGAG-3′ and antisense: 5′-TTGGGGTTGAGGGTTTTACA-3′ from bases 3969 to 4416), and type X collagen (sense: 5′-ACAAAGAGCGGACAGAGACC-3′ and antisense: 5′-AGAAGGACGAGTGGACATAC-3′ from bases 31 to 472) were prepared based on published DNA sequences (GenBank accession numbers J03485, L48440, and S79214, respectively). Primers for TGF-β type I receptor (TGF-βRI) (sense: 5′-GAAGAAGTTGCCGTGAAGATA-3′ and antisense: 5′-CGTGGACAGAGCGAGTTTGAT-3′ from bases 712 to 960), TGF-β type II receptor (TGF-βRII) (sense: 5′-GGAGCGGAAGACGGAGATGG-3′ and antisense: 5′-TGGATGCCCTGGTGGTTGAG-3′ from bases 991 to 1590), and BMP type IA receptor (BMPR-IA) (sense: 5′-CATTTCCAGCCCTACATCAT-3′ and antisense: 5′-TCTTTGCGAGCGTCTTCTTG-3′ from bases 1343 to 1652) were also prepared based on published DNA sequences (Genbank accession numbers L26110, L09653, and D38082, respectively). The specificity of these primers was assessed by the restriction enzyme digestion of their PCR products, which produced two specific products of the correct size. Primers for osteocalcin were prepared based on a previously described sequence [Araki et al., 1993]. Primers for actin were kindly provided by Dr. E.M. Greenfield (Department of Orthopaedics, Case Western Reserve University, Cleveland, OH). Amplifications were performed in a Robocycler Gradient 40 temperature cycler (Stratagene Cloning Systems, La Jolla, CA). DNA amplification included an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation

at 94°C for 1 min, annealing at 54°C (type I and X collagens), 56°C (type II collagen and aggrecan), 58°C (actin), or 60°C (TGF-βRI, TGF-βRII and BMPR-IA) for 1 min, and extension at 72°C for 1 min. The final cycle included 5 min for extension. The PCR products were analyzed by electrophoresis in 2% agarose gels stained with ethidium bromide. *Hae*III restriction fragments of ϕX174 DNA (Gibco BRL) were used as molecular weight markers.

Histological Analysis

The aggregates were harvested on Day 14, fixed in 10% neutral-buffered formalin (pH 7.0) for 2 h, embedded in a 1% agarose gel for ease of handling and fixed in 70% ethanol overnight. After dehydration, each gel containing an aggregate was embedded in paraffin and serial 5 μm sections were cut. Representative sections were stained with toluidine blue for the detection of cartilage matrix and replicate sections were used for immunohistochemistry.

Immunohistochemical Analysis

Immunohistochemistry for the expression of types II and X collagen was done by the method described previously [Johnstone et al., 1998; Yoo et al., 1998]. Briefly, following deparaffinization, the sections were treated with 0.1 U/ml chondroitinase in 1% BSA/phosphate-buffered saline (PBS) at room temperature (RT) for 30 min. After washing with PBS, the sections were blocked with 5% BSA/PBS for 30 min. Primary mouse anti-type II (II-II6B3, developed by Thomas Linsenmayer and obtained from the Developmental Studies Hybridoma Bank maintained by The University of Iowa, Department of Biological Science, Iowa City, IA, under contract NO1-HD-7-3263 from the NICHI) and anti-type X (kindly provided by Dr. Gary Gibson, Henry Ford Hospital, Detroit, MI) collagen monoclonal antibodies (1:100 dilution)/1% BSA/PBS were then applied to the sections and incubated at RT for 1 h. Following extensive washing with PBS, the sections were incubated in a 1:400 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin in 1% BSA/PBS at RT for an additional 1 h, rinsed again with PBS repeatedly, and mounted in a medium of glycerol: PBS (9:1) at pH 8.5 containing 0.01 M *para*-phenylenediamine (Eastman Kodak, Rochester, NY). Control sections were exposed to the identical staining sequence, except that the anti-collagen antibodies were substituted with a 1:100 dilution of normal mouse serum in 1% BSA/PBS. These sections were examined and photographed with an Olympus BH-2 fluorescence photomicroscope.

In Vivo Ceramic Cube Assay

The ceramic composite assay was performed to test the in vivo osteochondrogenic potential of the cultured cells [Goshima et al., 1991; Dennis et al., 1992; Nakahara et al., 1992; Cassiede et al., 1996]. At the end of secondary culture, periosteal cells were rinsed with Tyrode's solution, harvested by trypsinization, resuspended at 5×10^6 cells/ml in serum-free DMEM-LG medium, and placed in a 5-ml tube containing 3-mm porous calcium phosphate ceramics cubes (a generous gift from Zimmer/Bristol Myers Squib, Warsaw, IN), precoated with fibronectin (Collaborative Biomedical, Bedford, MA). After producing a vacuum to release air pockets from the ceramic cubes, the tubes were placed in a CO_2 incubator at 37°C for 2 h to allow the cells to attach to the ceramic surface. The cubes were then implanted subcutaneously into syngeneic F344 male rats. The ceramics were harvested 3 weeks post-implantation and fixed in 10% neutral-buffered formalin. After decalcification with RDO (Apex Engineering Products Co., Plainfield, IL), the ceramics were processed for routine histology. Sections were stained with toluidine blue and Mallory-Heidenhain's.

RESULTS

Osteochondrogenic Potential of Culture-Expanded Periosteal Cells

The culture-expanded periosteal cells were positively stained in the histochemical ALP activity assay of the monolayer cultures (data not shown). To examine the osteochondrogenic potential of the periosteal cells, they were loaded into the porous ceramic cubes and then implanted into syngeneic rats, as done previously with other cell preparations [Goshima et al., 1991; Dennis et al., 1992; Nakahara et al., 1992; Cassiede et al., 1996]. As presented in Figure 1, woven bone and cartilaginous tissue were formed. The cartilage consisted of hypertrophic chondrocytes, surrounded by matrix that stained metachromatically with toluidine blue. These data further suggested that cul-

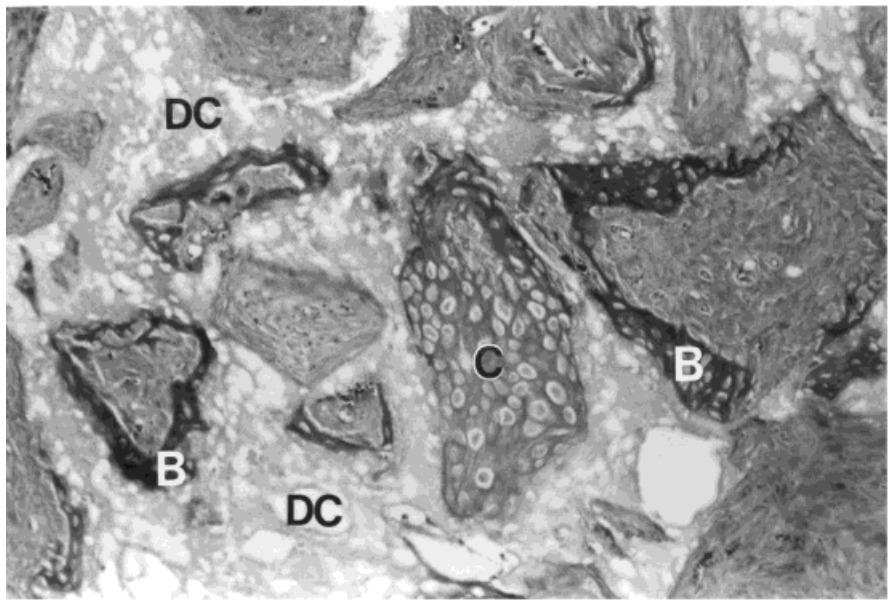

Fig. 1. Histologic features of a ceramic cube section loaded with periosteal cells (original magnification, 32×). Cubes were harvested from host rats 3 weeks postimplantation. After fixation and decalcification, histologic sections were prepared and stained with Mallory-Heidenhein's. Cartilaginous tissue (C) and woven bone (B) were formed along the walls of individual pores. Decalcified ceramic material (DC) appears as the lightly-stained acellular area.

ture-expanded periosteal cells possess osteochondrogenic potential.

Expression of Cartilage-Specific Genes in the Aggregate Cultures Treated With TGF-β1, BMP-2 or Both Factors

To investigate the effects of TGF-β1 and BMP-2 on the chondrogenic differentiation of aggregated periosteal cells, mRNA expression of aggrecan core protein and types I, II and X collagen was assessed by RT-PCR as shown in Figure 2. The mRNA of type I collagen was expressed in all aggregates on Day 3, but gradually decreased by Day 14. In contrast, aggrecan core protein mRNA expression was detected in the aggregates treated with TGF-β1+BMP-2 on Day 3, and increased on days 7 and 11. The aggregates still showed substantial expression on Day 14. BMP-2 treatment also induced aggrecan core protein mRNA expression by Day 7, and this appeared greater on Day 11. In TGF-β1-treated aggregates, aggrecan core protein gene expression was detected by Day 7, but it decreased with time. A detectable level of type II collagen mRNA was observed in BMP-2- and TGF-β1+BMP-2-treated aggregates on Day 7, and the expression was markedly increased on days 11 and 14. An expression of type X collagen mRNA was observed only in the BMP-2-treated aggregates on days 11 and 14; aggregates in the combined treatment with TGF-β1+BMP-2 did not express type X collagen gene throughout the experiment. Neither type II nor type X collagen genes were detected in either control or TGF-β1-treated aggregates at any time-point.

To corroborate these results, mRNA expression of collagens type II and X and osteocalcin, which is expressed by hypertrophic chondrocytes [Lian et al., 1993; Gerstenfeld and Shapiro, 1996], was examined on Day 14 in four different rat periosteal cell outgrowth preparations. Again, BMP-2 treatment induced mRNA expression of collagens type II and X, and osteocalcin, whereas TGF-β1+BMP-2-treated aggregates expressed neither type X collagens nor osteocalcin (Fig. 3). Neither of the collagen genes was expressed in the control or TGF-β1-treated aggregates and the results were consistent with those presented in Figure 2.

Histological and Immunohistochemical Analyses of the Chondrogenesis Induced by BMP-2 and TGF-β1+BMP-2

The 14-day aggregates treated with BMP-2 or TGF-β1+BMP-2 were consistently bigger than those treated with TGF-β1 or control. Control aggregates tended to disassociate into small fragments in the later stages of culture (Days 11–14). Histology revealed that many

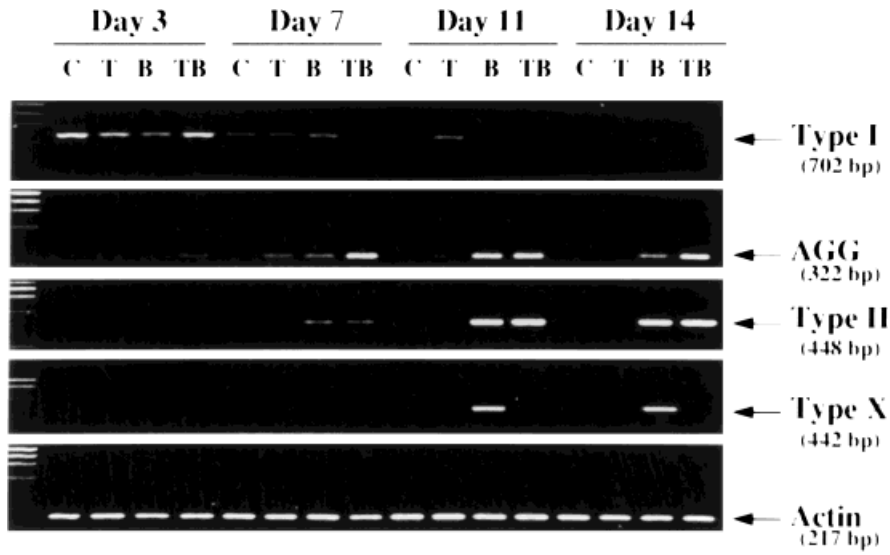

Fig. 2. RT-PCR analyses for mRNA expression of cartilage-specific matrix proteins in three-dimensional aggregate culture of rat periosteal cells. The aggregates were treated with no factors (control, C), 10 ng/ml TGF-β1 (T), 25 ng/ml BMP-2 (B), or a combination of TGF-β1 and BMP-2 (TB) in defined medium. RNA was extracted on days 3, 7, 11, and 14. Separate PCRs were done for type I (702 bp), II (448 bp) and X (442 bp) collagens, aggrecan core protein (AGG, 322 bp) and actin (217 bp). Reaction products were visualized on ethidium bromide-stained agarose gels. The first lane on the left represents molecular weight markers of *Hae*III restriction fragments of ϕX174 DNA.

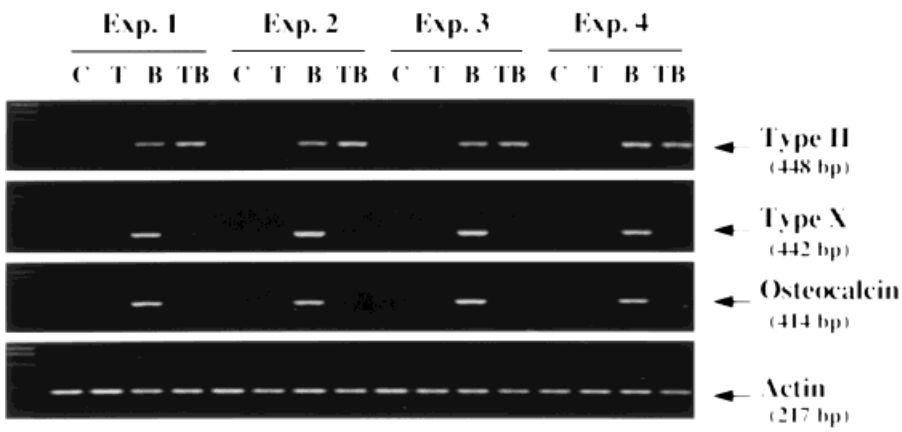

Fig. 3. RT-PCR analyses for mRNA expression of cartilage-specific matrix proteins on Day 14. The aggregates were treated with no factors (control, C), 10 ng/ml TGF-β1 (T), 25 ng/ml BMP-2 (B), or a combination of TGF-β1 and BMP-2 (TB) in defined medium. Four independent experiments were performed and RNA was extracted from the aggregate cultures on Day 14. Separate PCRs were done as described in the legend for Figure 2 except for additional assessment of osteocalcin (414 bp).

hypertrophic chondrocytes with metachromatic matrix appeared in BMP-2-treated aggregates on Day 14. In contrast, TGF-β1+BMP-2-treated aggregates had an abundant metachromatic matrix in which many chondrocytes were embedded, but hypertrophic chondrocytes were seldom seen (Fig. 4). Neither chondrocytes nor cartilaginous matrix was observed in control or TGF-β1-treated aggregates.

To demonstrate the formation and matrix deposition of type II and X collagens, immunohistochemistry was performed on sections of BMP-2- and TGF-β1+BMP-2-treated aggregates using specific monoclonal antibodies. Immunoreactivity to anti-type II antibody was localized in the cartilaginous matrix of 14 day aggregates treated with BMP-2 and TGF-β1+BMP-2 (Fig. 5). However, positive immunoreactivity to type X collagen was found only in BMP-2-treated aggregates (Fig. 5). The immunofluorescence was localized to the matrix areas surrounding the hypertrophic chondro-

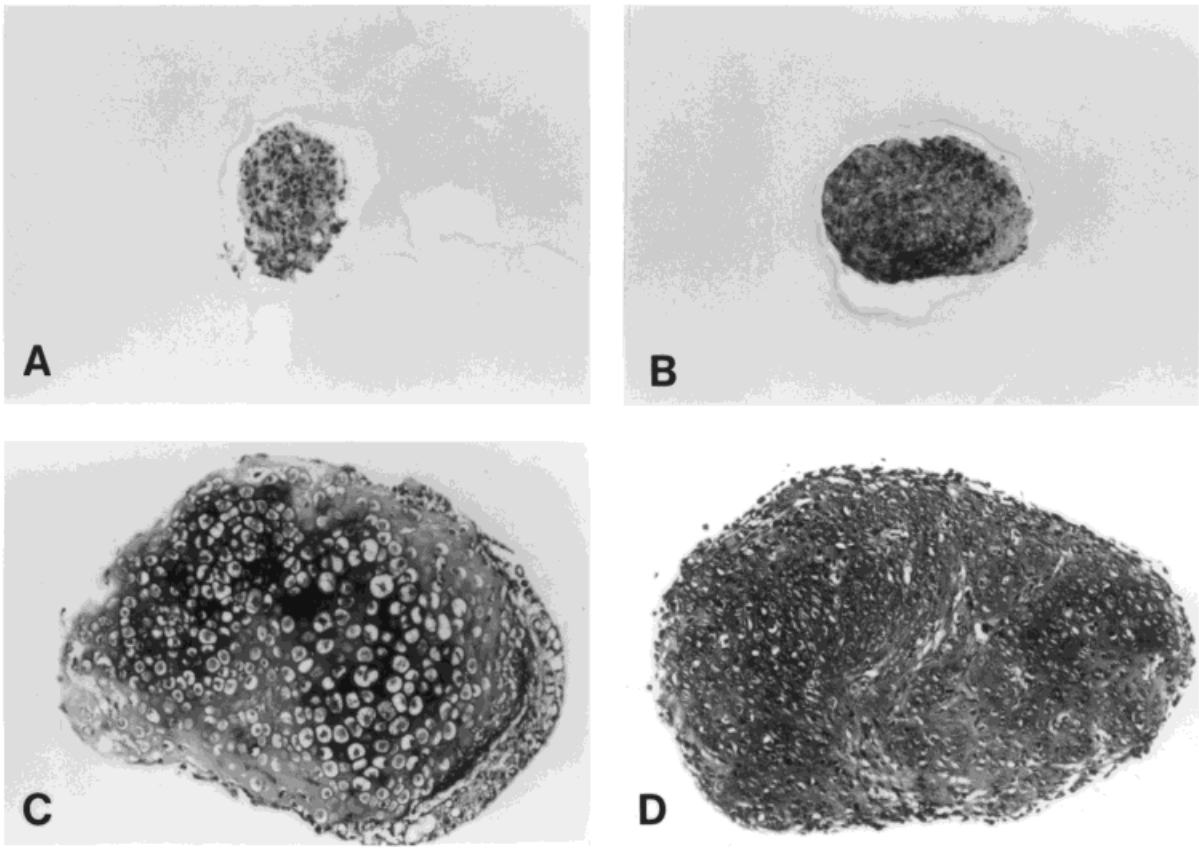

Fig. 4. Histology of toluidine blue-stained aggregates on Day 14 (original magnification, 32×). The aggregates were treated with no factors (control, **A**), 10 ng/ml TGF-β1 (**B**), 25 ng/ml BMP-2 (**C**), or a combination of TGF-β1 and BMP-2 (**D**) in defined medium. Many mature and hypertrophic chondrocytes appeared in the aggregates treated with BMP-2. In contrast, hypertrophic chondrocytes were seldom seen in aggregates treated with TGF-β1 and BMP-2.

cytes. Type X collagen could not be detected immunohistochemically in aggregates treated with TGF-β1+BMP-2.

Continuous Expression of BMP-2 and TGF-β1 Receptor Genes in the Aggregate Cultures

To examine whether the lack of response of aggregated cells to TGF-β1 was due to a lack of receptor expression, we examined the mRNA expression of TGF-βRI and RII and BMPR-IA receptors of control, TGF-β1 and BMP-2-treated aggregates. BMPR-IA and TGF-βRI and RII mRNA expression was detected in the aggregates. There was some variation in band density, but expression was detected in all treatment groups throughout the culture period (Fig. 6).

DISCUSSION

The periosteum contains several cell types in the outer layer and precursor cells in the inner cambium layer that support osteoblastic and chondrogeneic differentiation during bone formation and growth [Izumi et al., 1992; Taylor, 1992; Iwasaki et al., 1993, 1994; O'Driscoll et al., 1994]. We successfully introduced ALP-positive osteochondral progenitor cells into in vitro culture by a periosteum explant outgrowth method; a small percentage of ALP-negative cells was also present. The latter may be fibroblasts from the outer layer or undifferentiated mesenchymal progenitor cells. The in vivo ceramic cube implantation assay demonstrated that the cultured rat periosteal cells had osteochondrogenic potential. To study this in vitro, three-dimensional aggregate cultures of these cells were performed in the presence and absence of chondroinductive agents. Chondrogenesis was only detected when BMP-2 was exogenously added to the defined medium.

The pattern of gene expression during chondrogenesis in vitro of BMP-2-treated rat peri-

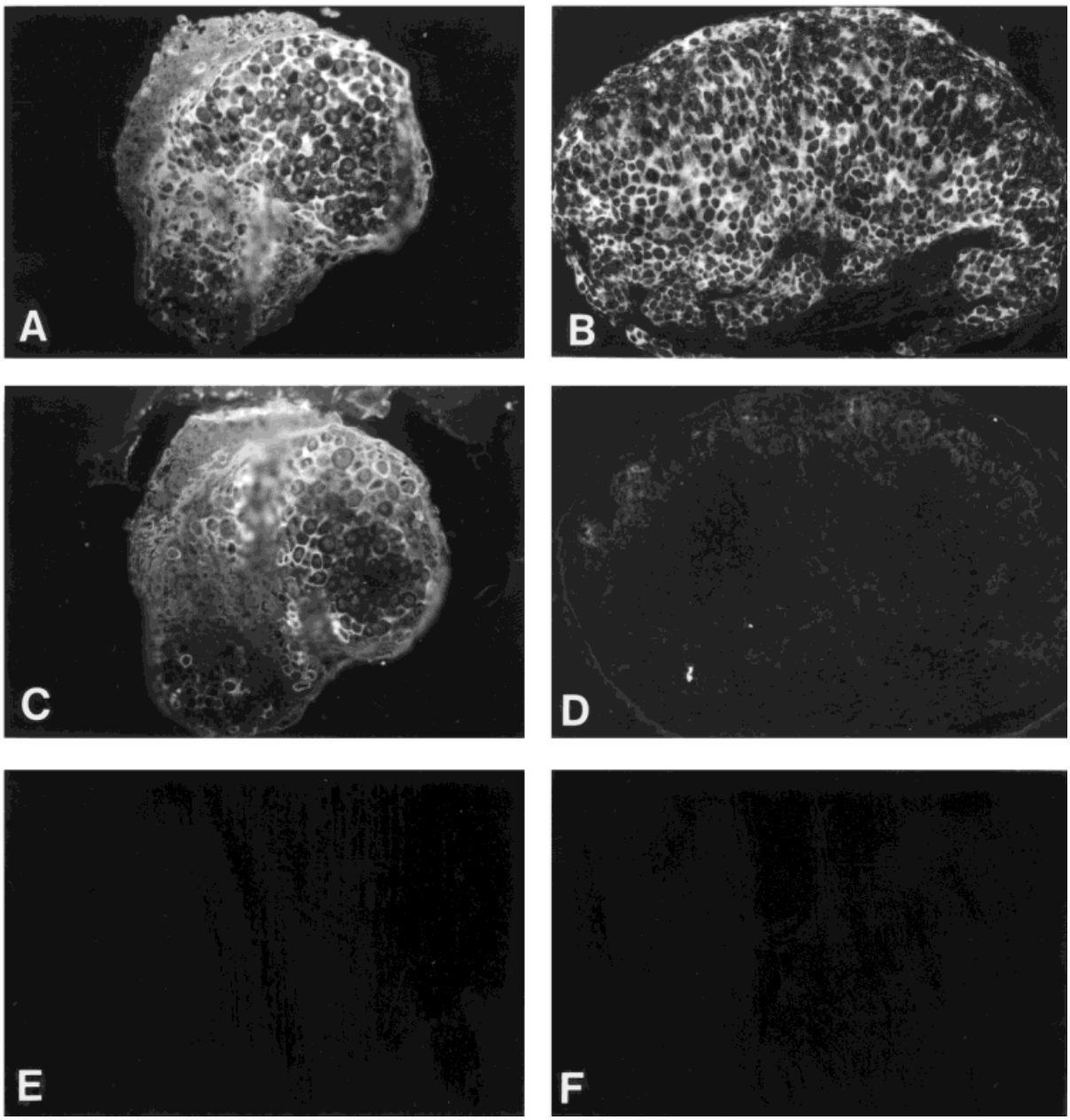

Fig. 5. Immunohistochemical staining of 14-day aggregates (original magnification, 32×). The aggregates treated with 25 ng/ml BMP-2 (**A**, **C**) or a combination of TGF-β1 and BMP-2 (**B**, **D**) were fixed and embedded in paraffin. Sections of BMP-2-treated aggregate had detectable expression of both type II (A) and X (C) collagens in the matrix. Note that TGF-β1+BMP-2 treated aggregates had intense staining for type II collagen (B) in the matrix, but no type X collagen (D) was detected. Sections of the BMP-2- or TGF-β1+BMP-2 treated aggregates stained with normal serum instead of primary antibodies were done as controls (**E** and **F**, respectively).

osteal cells has features in common with that observed for other cell progenitor cell types in similar culture conditions [Kosher et al., 1986; Edwall-Arvidsson and Wroblewski, 1996; Shukunami et al., 1996]. At the earliest stages, the mesenchymal precursor cells produce type I collagen. After the stage of precartilaginous condensation, a switch occurs in collagen synthesis from type I to type II collagen. Type I collagen synthesis decreases, while the level of type II collagen transcripts increases. Differentiating chondrocytes then express cartilage specific marker transcripts, namely aggrecan and type II collagen. As differentiation con-

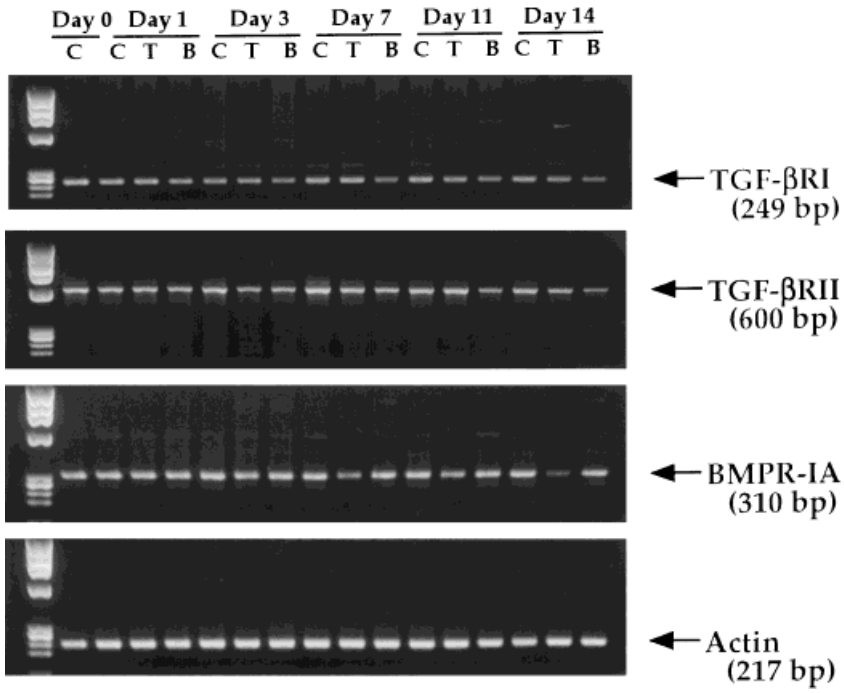

Fig. 6. RT-PCR analyses for mRNA expression of TGF-β1 type 1 (TGF-βRI) and type 2 (TGF-βRII), and BMP type 1A (BMPR-IA) receptors in three-dimensional aggregate culture of rat periosteal cells. The aggregates were treated with no factors (control, C), 10 ng/ml TGF-β1 (T), or 25 ng/ml BMP-2 (B) in defined medium. RNA was extracted on days 0, 1, 3, 7, 11, and 14. Separate PCRs were done for TGF-βRI (249 bp), TGF-βRII (600 bp), BMPR-IA (310 bp), and actin (217 bp). Reaction products were visualized on ethidium bromide-stained agarose gels. The first lane on the left represents molecular weight markers of *Hae*III restriction fragments of ϕX174 DNA.

tinues toward hypertrophy, type X collagen is synthesized [Cancedda et al., 1995]. In aggregated rat periosteal cells treated with BMP-2, type I collagen mRNA expression was detected on Day 3, but decreased thereafter. In contrast, aggrecan core protein and type II collagen mRNA were detectable by Day 7 and expressed on Day 11. Type X collagen mRNA was detectable by Day 11. Histological and immunohistochemical studies indicated that the aggregates contained hypertrophic chondrocytes with both type II and X collagens being localized in the cartilage extracellular matrix of the aggregates on Day 14. The development of chondrocyte hypertrophy is accompanied by the expression of matrix proteins generally considered as markers of the calcified cartilage as well as the osteoblastic phenotype, such as osteocalcin [Lian et al., 1993; Cancedda et al., 1995; Gerstenfeld and Shapiro, 1996]. Osteocalcin mRNA was detected in 14-day BMP-2-treated aggregates. Thus, this culture system model offers the opportunity to follow the temporal molecular sequence of events underlying in vitro chondrogenesis from the progenitor cell stage to a fully differentiated phenotype.

In cultures of embryonic chick limb bud cells, TGF-β1 treatment induced the formation of cartilaginous tissue [Roark and Greer, 1994]. However, TGF-β1 failed to induce chondrogenesis in the rat periosteal cell aggregates. It is unlikely that the unresponsiveness of aggregates to TGF-β1 is due to a lack of its receptors, since all aggregates continuously expressed mRNA for TGF-βRI and RII receptors (Fig. 6). Rabbit periosteal explants undergo chondrogenesis when exposed to TGF-β1 [O'Driscoll et al., 1994; Nishimura et al., 1999], indicating that this may be a difference in the type of culture used. However, this may not be the full explanation: although rabbit and human bone marrow-derived progenitor cell aggregates undergo chondrogenesis in response to TGF-β1+dexamethasone [Johnstone et al., 1998; Yoo et al., 1998], rat marrow-derived cells do not (Solchaga LA, Yoo JU, Johnstone B, unpublished results), indicating a species difference in response.

TGF-β1 inhibited the terminal differentiation to hypertrophic chondrocytes of the BMP-2-treated periosteal cells, as evidenced by the absence of mRNA expression for type X collagen and osteocalcin and our inability to detect type X collagen production at the protein level, as assessed by immunohistochemistry. This inhibitory effect of TGF-β1 was previously observed in aggregate cultures of rat growth plate chondrocytes [Kato et al., 1988], in which TGF-β1 delayed hypertrophy of the differentiating chondrocytes in low serum or defined medium conditions. In the present study, TGF-β1+ BMP-2-treated aggregates showed detectable expression of aggrecan core protein mRNA on Day 3 and continued expression for days 7–14. Gene expression of type II collagen was also pronounced for days 11–14. Histological and immunohistochemical analyses showed that the TGF-β1+BMP-2-treated aggregates consisted of chondrocytes and abundant cartilage matrix that stained with anti-type II collagen antibody. Thus, TGF-β1 appeared to stabilize the phenotype of pre-hypertrophic chondrocytes by stimulating the expression of aggrecan and type II collagen, and by inhibiting that of type X collagen. The chondrogenic differentiation of periosteal cells in vivo may be controlled by the balance of action of autocrine or paracrine factors such as TGF-β1 and BMP-2.

BMP-2 induces chondrogenic differentiation of C3H10T1/2 cells [Wang et al., 1993] and facilitates the cartilage formation during endochondral ossification in ectopic bone formation [Wozney et al., 1988]. BMP-2 is upregulated in periosteal explant cultures that undergo chondrogenesis, and its expression is enhanced by addition of TGF-β1 to the cultures [Sanyal et al., 1999]. BMP-2 and its receptors type IA (ALK-3) and IB (ALK-6) are expressed in periosteal cells of fracture callus [Bostrom et al., 1995; Ishidou et al., 1995]. BMP-2 is also stored in bone matrix and may be exposed to cells including periosteal and bone marrow-derived mesenchymal progenitor cells when bones are fractured [Rosen and Thies, 1992]. These results suggest that BMP-2 plays an important role in rat periosteum-derived chondrogenesis. Nevertheless, the exact mechanism governing periosteal chondrogenesis is still unclear. The present culture system may be a useful model for the further characterization of the neochondrogenesis of periosteal cells.

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