collagen matrix with BMP-2 and BMP-7 generate new bone as well as, or better than, autografts of healthy bone transplanted from another part of the patient's body. However, extremely high quantities of BMPs are needed to produce bone matrix, which is a major limitation to this approach (Service, 2000).

A third element required for in vivo bone formation, in addition to stem cells and cytokines, is a matrix or scaffold (Chen et al., 2000a,b). In an attempt to use the stromal system to direct clinical osteogenesis, marrow stromal cells implanted on gelatin sponges were shown to repair a craniofacial defect (Krebsbach et al., 1998; Tabata et al., 2000). Stromal cells implanted on hydorxyapatite/tricalcium phosphate ceramics also regenerated a critical size defect in the tibia, and resulted in consistent bone formation (Krebsbach et al., 1997). Various types of biomaterials as a scaffold is necessary for the successful tissue engineering (Ohgushi and Caplan, 1999).

In the present study, we show that clonal stromal cells can generate bone of custom-shapes and sizes in combination with an appropriate scaffold, in this case poly-DL-lactic-co-glycolic acid (PLGA)-collagen sponge.

MATERIALS AND METHODS

Cell culture

Primary culture of the marrow cells was performed according to Dexter's method (Dexter et al., 1977). Female C3H/He mice (n = 10) were anesthetized with ether, femurs were excised, and bone marrow cells were obtained for primary bone marrow cultures. Cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal bovine serum (FBS) and penicillin (100 μg/ml)/streptomycin (250 ng/ml) at 37°C in humid air with 5% CO_2. Immortalized cells were obtained by frequent subculture for more than a year. Cell lines from different dishes were subcloned by limiting dilution. The screened line named KUSA/A1 was cultured in the maintenance medium consisting of IMDM supplemented with 10% FBS and penicillin (100 μg/ml)/streptomycin (250 ng/ml) at 37°C in humid air with 5% CO_2 as previously described (Umezawa et al., 1992). As for monolayer culture, KUSA/A1 cells was cultured with 50 μg/ml ascorbic acid and 10 mM sodium β-glycerophosphate in their culture media.

In vitro calcification assay and osteocalcin production

Cells were sonicated in the homogenization-buffer [20 mM Tris/HCl (pH 7.2), 0.1% Triton X-100] after washing twice with PBS. Calcium content in the culture was determined by CalciumC Test (Wako Chemical Co., Japan) according to the manufacturer's suggestion. The amount of osteocalcin into the culture media was determined by RIA using a mouse osteocalcin assay kit (Biomedical Technologies Inc., Stoughton, MA).

Measurement of alkaline phosphatase (ALP) and evaluation of parathyroid hormone (PTH) response

KUSA/A1 cells were analyzed by ALP assay as described (Leboy et al., 1991). PTH response was evaluated in KUSA/A1 cells as described (Sato et al., 1987).

Cytochemical and histochemical staining for ALP activity and β-galactosidase

Cells in 35 mm dishes were maintained in the standard media described above. Fixed cultures and sections were stained for 30 min at 37°C using naphthol AS-MX phosphate as a substrate and fast red violet LB salt as a coupler. Some cultures were maintained with standard media supplemented with 50 μg/ml ascorbic acid and 10 mM sodium β-glycerophosphate for up to 30 days in vitro. Sections were also stained with Xgal for 8 h at 37°C. Cells expressing β-galactosidase show a blue color after incubation with the Xgal substrate. In our hands, color formation was evident by 10–30 min incubation.

RNA extraction and Northern blotting

RNA was prepared by homogenizing the specimens in guanidinium isothiocyanate followed by centrifugation over a cesium chloride cushion as previously described (Umezawa et al., 1991). The RNA was then electrophoresed in a 1.0% agarose gel, transferred to a nylon filter (NEN Research Products, Boston MA), and hybridized with a murine collagen α2(I) (TIE5) cDNA probe (Amagai et al., 1989). The probe was labeled with [α-^{32}P]-CTP by the random-primer method (Feinberg and Vogelstein, 1983). Hybridization was carried out at 65°C for 14–16 h in a buffer containing five times SSPE (one time SSPE is 0.18 M NaCl, 10 mM NaH_2PO4/Na_2HPO_4 <pH 7.0>, pH 7.4, 1 mM EDTA), five times Denhardt's solution (one time Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.02% poly A, and 1% sodium dodecyl sulfate (SDS). The blots were washed with one time SSC containing 1% SDS at 65°C. The blots were exposed to X-ray film at −80°C using an intensifying screen.

Three dimensional collagen gel culture

KUSA/A1 cells were dispersely embedded in type I collagen gels. Eight volumes of cooled collagen solution (I-A Cellmatrix, Nitta gelatin, Osaka, Japan), one volumes of ten times concentrated IMDM medium, and one volume of buffer solution (0.05 N NaOH containing 2.2% $NaHCO_3$ and 200 mM HEPES) were added with gentle shaking on ice to ten volumes of IMDM medium suspension containing 10^7 cells/ml KUSA/A1 cells. Two hundred microliters of this medium-cell-collagen mixture was placed in each well of 96 well plastic microwell plate and allowed to gel by incubation at 37°C for 30 min. The gels were transferred to a 10 mm plastic dish and overlaid with 10 ml of IMDM containing 10% FBS. Cell cultures were incubated in 5% CO_2 at 37°C and the medium was changed every 3 days.

Introduction of the β-galactosidase gene

Recombinant adenovirus expressing bacterial β-galactosidase was prepared as described (Yamashita et al., 1999). Cells were infected by this virus at 10 plaque forming unit/cell. The expression of the β-galactosidase gene in KUSA/A1 was determined cytochemically in vitro. Nearly all the cells expressed β-galactosidase 3 days after transfection.

Inoculating of cells into syngeneic mice

Culture medium containing either KUSA/A1 cells (10^7 cells/ml) was injected to 8–12 weeks old female

C3H/He mice subcutaneously. The tumors were harvested and fixed in 20% formalin 1 day to 8 weeks after the inoculation. Serial paraffin sections were prepared and stained with either hematoxylin and eosin (H&E), von Kossa or Toluidine Blue stain.

In vivo culture in diffusion chamber

Paired diffusion chambers were constructed using lucid rings and 0.45 μm membrane filters (Millipore Corporation, Bedford, MA) (Urist et al., 1982). The chambers shared a common filter. One chamber contained either KUSA/A1 cells in 200 μl aliquots from six dishes of confluent cultures. After receiving the contents, chambers were implanted subcutaneously in 8–12 weeks old female C3H/He mice. The animals were sacrificed after 2–8 weeks. The chambers were fixed in 20% formalin. Serial paraffin sections were prepared and stained with either hematoxylin and eosin, von Kossa or Toluidine Blue stain.

Preparation of cells, staining procedures, and fluorescence activated cell analysis

All samples (KUSA/A1) were treated by water lysis. Cells, at a final concentration of 1×10^7/ml, were incubated with 1 μg/μl monoclonal antibodies in Hank's balanced salt solution (containing 0.1% albumin and 0.1% sodium azide). In case that the first antibody is conjugated with biotin, cells were washed twice and incubated with streptoavidin-phycoerythrin (Gibco-BRL, Rockville, MD) for 30 min on ice. Purified antibodies in the first step were stained with FITC conjugated goat anti-mouse antibody. Antibodies (anti-mouse Flk-1, CD31, CD34, c-kit, Sca-1, CD140a, CD144, CD14, CD29, CD41, CD44, CD49b, CD49d, CD54, CD90, CD102, CD106, Ly-6C and Ly-6G, and isotype control antibodies) were purchased from Pharmingen Pharmaceutical, Inc. (San Diego, CA). After two washes with Hank's balanced salt solution, propidium iodide (PI) was added to each test tube at a concentration of 1 mg/ml just before acquisition by FACScan flow cytometry (Beckton Dickinson, Franklin Lakes, NJ) with the Argon laser at 488 nm. List mode data for 30,000–50,000 cells were collected in PI gate.

PLGA-collagen hybrid sponge preparation

Biodegradable hybrid sponge of PLGA (a 75:25 copolymer of lactic acid and glycolic acid with molecular weight of 90–126 kDa) and collagen was used as the three-dimensional porous scaffold for implantation (Chen et al., 2000a,b). The hybrid sponge was prepared by forming microsponges of collagen in the pores of PLGA sponge. At first, a PLGA sponge was prepared by a particulate-leaching technique using sieved sodium chloride particulates. PLGA polymer was dissolved in chloroform to prepare a PLGA solution in chloroform at a concentration of 20% (w/v). Sieved NaCl particulates (9.0 g), ranging in diameter from 355 to 425 μm, were added to the PLGA solution (5 ml), vortexed, and poured into an aluminum pan. The chloroform was allowed to evaporate by air-drying in a draft for 24 h and followed by 24 h of vacuum drying. The PLGA sponge with porosity of 90% and pore size of 355–425 μm was formed after NaCl particulates were leached out by washing with deionized water. Subsequently, the PLGA sponge was immersed in a bovine collagen acidic solution (type I, pH 3.2, 5 μg/μl) under a vacuum so that the sponge pores filled with collagen solution. The collagen solution containing PLGA sponge was then frozen at −80°C for 12 h and lyophilized under a vacuum of 0.2 Torr for an additional 24 h to allow the formation of collagen microsponges in the sponge pores. Finally, the collagen microsponges were cross-linked by treatment with glutaraldehyde vapor saturated with 25% glutaraldehyde aqueous solution at 37°C for 4 h; and non-reacting aldehyde groups were blocked by treating with 0.1 M glycine aqueous solution. After washing with deionized water and lyophilizing, the PLGA-collagen hybrid sponge was prepared. The formation of collagen microsponge in the pores of PLGA sponge was confirmed by SEM observation. The hybrid sponge was sterilized using ethylene oxide.

Transplantation of cells in the PLGA sponge

KUSA/A1 suspension (10^7/ml) was injected gently into PLGA sponges. After injection, the sponges were incubated at 37°C for more than 30 min. Then the sponges were transplanted into subcutaneous tissue or abdominal cavity of mice. These operation plans are accepted by the Laboratory animal care and use committee of Keio University School of Medicine (#000024). All the experiments were followed by the guideline for the care and use of laboratory animals of Keio University School of Medicine.

RESULTS

Characterization of isolated differentiated osteoblasts

KUSA/A1 cells were originally isolated as cells which induce hematopoiesis in vivo (Umezawa et al., 1992). When cultured in monolayer, the cells were spindle-shaped in the growth phase and after confluence (Fig. 1A,B). Extracellular matrix positive for von Kossa stain appeared in cell culture and increased after confluence (Fig. 1C,D). Ultrastructurally, the matrix was electron dense and was clearly produced by the cells (Fig. 1E). RNA blot hybridization showed that the collagen α2(I) gene was expressed at a high level in KUSA/A1 cells, the same as in other stromal cell lines (Fig. 1F) (Umezawa et al., 1992).

We analyzed growth curve, ALP activity, in vitro calcification, osteocalcin (bone gla protein) release, and response to PTH in comparison with MC3T3-E1 cells. KUSA/A1 proliferation, which was measured by DNA content, was equivalent to that of MC3T3-E1 cells. ALP activity of KUSA/A1 cells in growth phase was approximately tenfold higher than in MC3T3-E1 cells (Fig. 1G). In vitro calcification, which steadily increased both before and after confluence, was approximately 100-fold higher after the confluent stage in KUSA/A1 cells than in MC3T3-E1 cells (Fig. 1H). Osteocalcin release into culture media peaked on day 5 in KUSA/A1 cells and was two- to threefold higher than in MC3T3-E1 cells (Fig. 1I). PTH response, measured as cAMP production, gradually decreased during the culture period in KUSA/A1 cells but increased in MC3T3-E1 cells (Fig. 1J). This response to PTH did not require any induction and was independent of culture conditions.

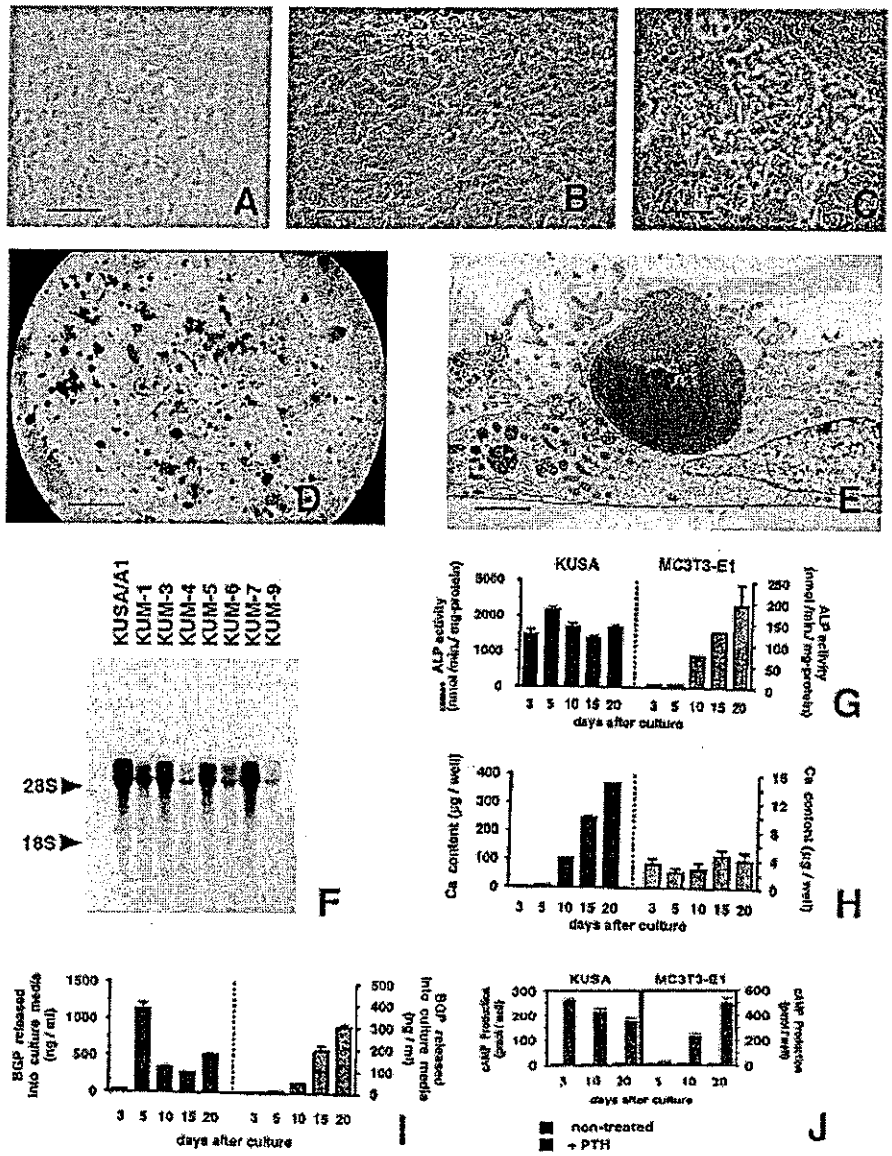

Fig. 1. In vitro characteristics of KUSA/A1 cells as a mature osteoblast model. A: Phase contrast micrograph of KUSA/A1 cells at the semiconfluent stage. B: KUSA/A1 cells 7 days postconfluence. C: Extracellular matrix produced from KUSA/A1 cells. D: Calcium deposition in KUSA/A1 culture. KUSA/A1 cells cultured in supplemented medium containing β-glycerophosphate were fixed and stained in situ by the von Kossa technique. E: Transmission electron micrograph of the extracellular matrix produced by KUSA/A1. F: Expression of the collagen α2(I) gene in marrow stromal cell lines (Umezawa et al., 1992). G: ALP activity in KUSA/A1 cells and MC3T3-E1 cells. H: Quantitative analysis of calcium deposition in KUSA/A1 cells and MC3T3-E1 cells. I: Bone Gla protein (osteocalcin) secretion in KUSA/A1 cells and MC3T3-E1 cells. J: cAMP production after PTH treatment. PTH response of KUSA/A1 cells was compared with that of MC3T3-E1 cells, as measured by cAMP production. Scale bars: 120 μm (A,B,C), 5 mm (D), 2 μm (E).

Three-dimensional culture of KUSA/A1 cells in collagen gels

To generate a three-dimensional cell culture system, KUSA/A1 cells were suspended and cultured in collagen gels (Fig. 2). Gels containing KUSA/A1 were transparent in vitro under a phase contrast microscopy on day 3 and remained semi-transparent until 2 weeks. Histologically, KUSA/A1 cells formed a reticular network, which had evidence of calcification by von Kossa staining at 2 weeks. These cells exhibited high ALP activity. At 4 weeks, heavy calcium deposition had rendered the gels opaque, even though the cultures were not supplemented with β-glycerophosphate.

In vivo osteogenic activity in KUSA/A1 cells

To determine the potential osteogenic activity of KUSA/A1 in vivo, the cells were injected subcutaneously into syngeneic mice (Fig. 3A–E). Before inoculation,

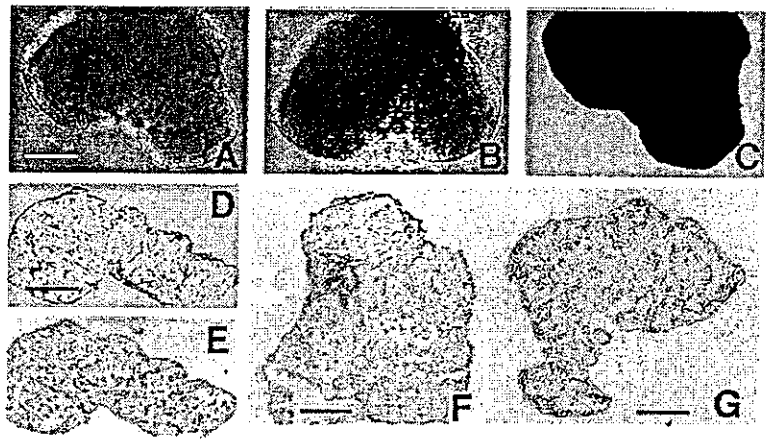

Fig. 2. Three-dimensional culture of KUSA/A1 cells in collagen gel. Phase contrast micrograph of three-dimensional culture of KUSA/A1 cells in collagen gel for 3 days (A), 2 (B), and 4 weeks (C). At 4 weeks, gels have become opaque. D: Microscopic examination of KUSA/A1 in collagen gels at 2 weeks. H&E stain. KUSA/A1 cells connected with each other and formed a reticular network. E: KUSA/A1 culture in collagen gels with von Kossa staining. The cells were cultured without β-glycerophosphate. F: ALP cytochemical staining of KUSA/A1 cells at 2 weeks. G: von Kossa staining of KUSA/A1 culture at 4 weeks. Scale bars: 170 μm (A,D,F,G).

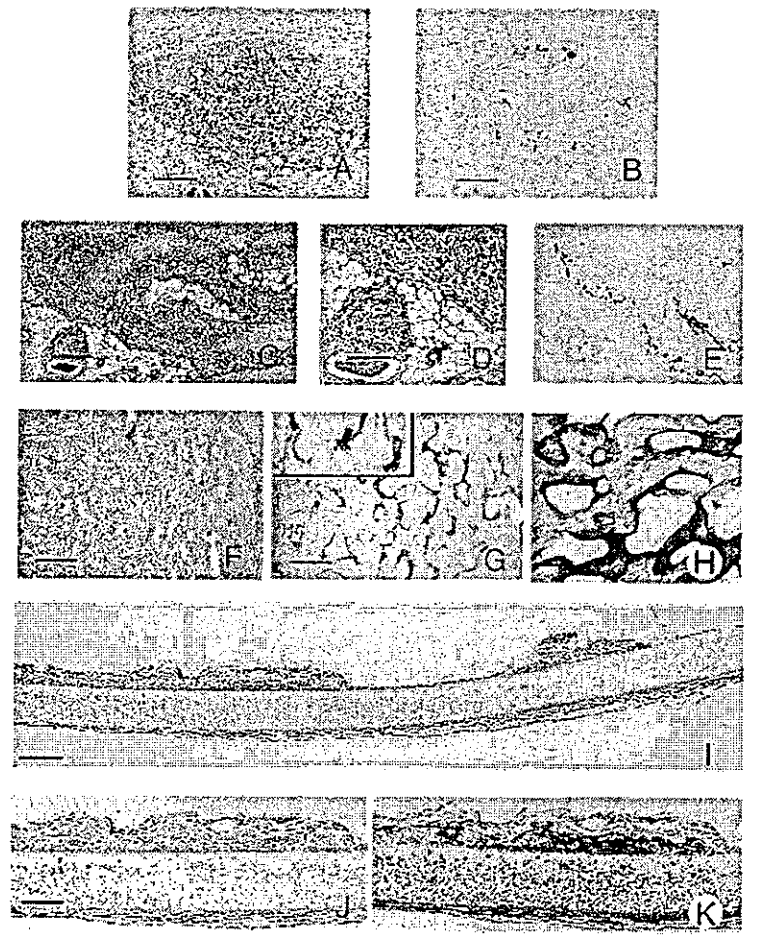

Fig. 3. Membranous ossification by KUSA/A1 in vivo. A and B: Microscopic examination of KUSA/A1 tumor in the subcutaneous tissue 2 days after injection. H&E stain (A) and von Kossa stain (B). Bone matrix was detected on day 2. C: KUSA/A1 tumor on day 3. H&E stain. D: Higher magnification of C. E: KUSA/A1 tumor on day 3 stained with von Kossa stain. Bone matrix had increased on day 3, as compared with day 2. F: Complete bone formation with a marrow cavity at 8 weeks. G: Enzyme histochemistry for β-galactosidase of KUSA/A1 tumor, 2 weeks after inoculation. Bone was generated by KUSA/A1 cells, which were transfected with β-galactosidase. H: Enzyme histochemistry for ALP of KUSA/A1 tumor, 2 weeks after injection. KUSA/A1 cells, which induce bone, were strongly positive for ALP. I, J, and K: In vivo diffusion chamber analysis of KUSA/A1 cells. Bone formation was detected not only inside the chamber but also outside the chamber, 4 weeks after transplantation. Note that calcium deposition was also observed outside the membrane as well as inside the membrane. I and J: H&E stain. K: von Kossa stain. Scale bars: 160 (A and B), 340 (C), 160 (D), 340 (E), 400 (F), 290 (G), 150 (G, inlet), 60 (H), 90 (I), 140 μm (J and K).

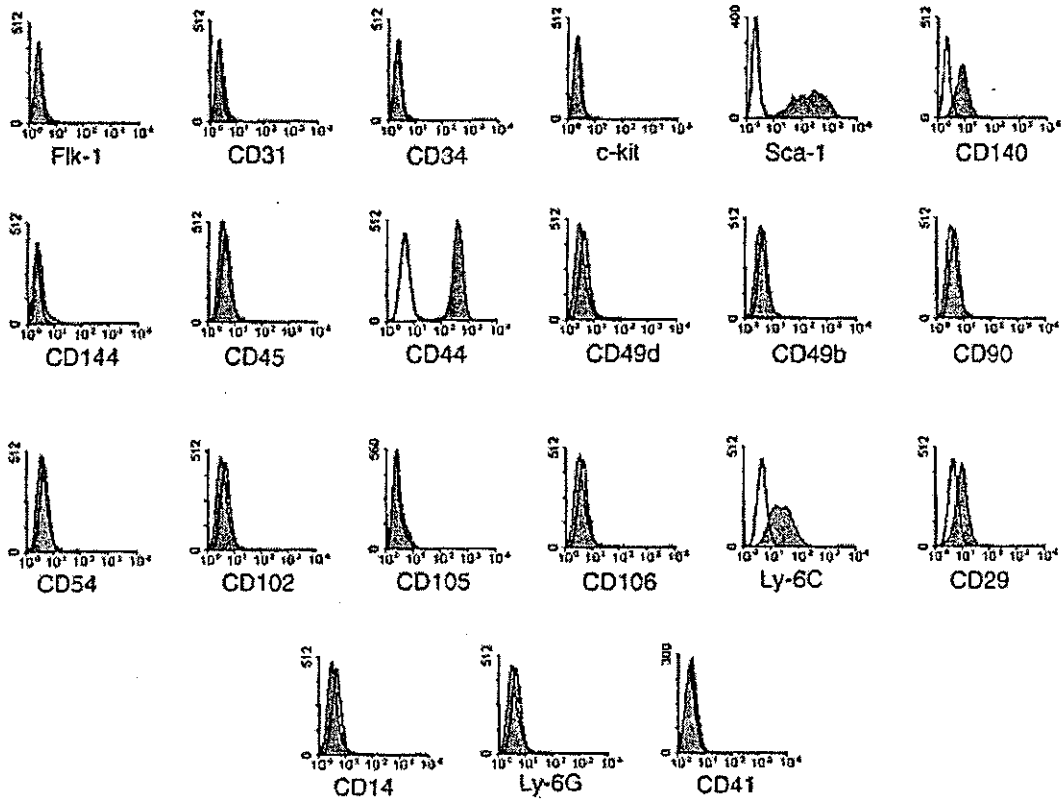

Fig. 4. Flow cytometry analysis of cell surface markers in KUSA/A1 cells. Cells stained with primary antibodies to Flk-1, CD31, CD34, c-kit, Sca-1, CD140, CD144, CD45, CD44, CD49d, CD49b, CD90, CD54, CD102, CD105, CD106, Ly-6C, CD29, CD14, Ly-6G, and CD41, are shown as gray peaks. The isotope controls are shown as white peaks.

cells were cultured for more than 5 days postconfluence. The inoculated cells were negative for calcium deposition on day 1 after the inoculation. But, bone matrix positive for von Kossa stain appeared in the injected cells on day 2, and had markedly increased and become larger, and the matrix became strongly positive for von Kossa stain on day 3. Marrow cavities were formed inside the bone generated by KUSA/A1 cells at 8 weeks and at later stages after inoculation. Hematopoietic cells were also observed in these bone marrow, as reported previously (Umezawa et al., 1992). No cartilage was formed in any KUSA/A1-transplanted bones, indicating that osteogenesis by KUSA/A1 is membranous ossification rather than enchondral ossification.

We continued to observe the fate of KUSA/A1 bone transplanted into the subcutaneous tissue and abdominal cavity, and found that the ectopic KUSA/A1 bone remained unchanged in size and shape for 12 months at both sites. Histologically, complete functional hematopoiesis by ectopic KUSA/A1 bone was seen in the subcutaneous tissue but not in the abdominal cavity.

Osteogenesis was directly generated by the inoculated KUSA/A1 osteoblasts

To determine whether these bones were directly formed by KUSA/A1 cells or generated by host-derived cells, we labeled KUSA/A1 cells with bacteria-derived β-galactosidase (Fig. 3F–H). Frozen tissue sections showed that osteoblasts in the bone were positive for β-galactosidase. The osteoblastic cells, which exhibited strong β-galactosidase activities were also strongly positive for ALP. Sarcomatous proliferation of the inoculated cells, which were positive for β-galactosidase was not seen around the generated bone.

We also injected KUSA/A1 cells into a diffusion chamber and examined osteogenesis (Fig. 3I–K). This was performed to determine whether these bones were directly formed by KUSA/A1 cells or generated by host-derived cells, and whether osteogenesis requires direct cell–cell interactions with the adjacent cells. Diffusion chambers containing KUSA/A1 cells were transplanted subcutaneously into mice. Bone was formed inside the chamber 2 weeks after the transplantation and had calcium deposition. This bone formation became more evident from 4 weeks after the inoculation. Surprisingly, we observed bone formation even outside the chamber in specimens taken at 4 weeks. Calcification was also seen both inside and outside the chamber, suggesting that remarkably strong osteogenic soluble factors or cytokines were being secreted from KUSA/A1 cells. Interestingly, calcium deposition was also reproducibly detected in the membrane. Therefore, osteogenesis is induced directly by KUSA/A1 cells rather than indirectly by neighboring cells.

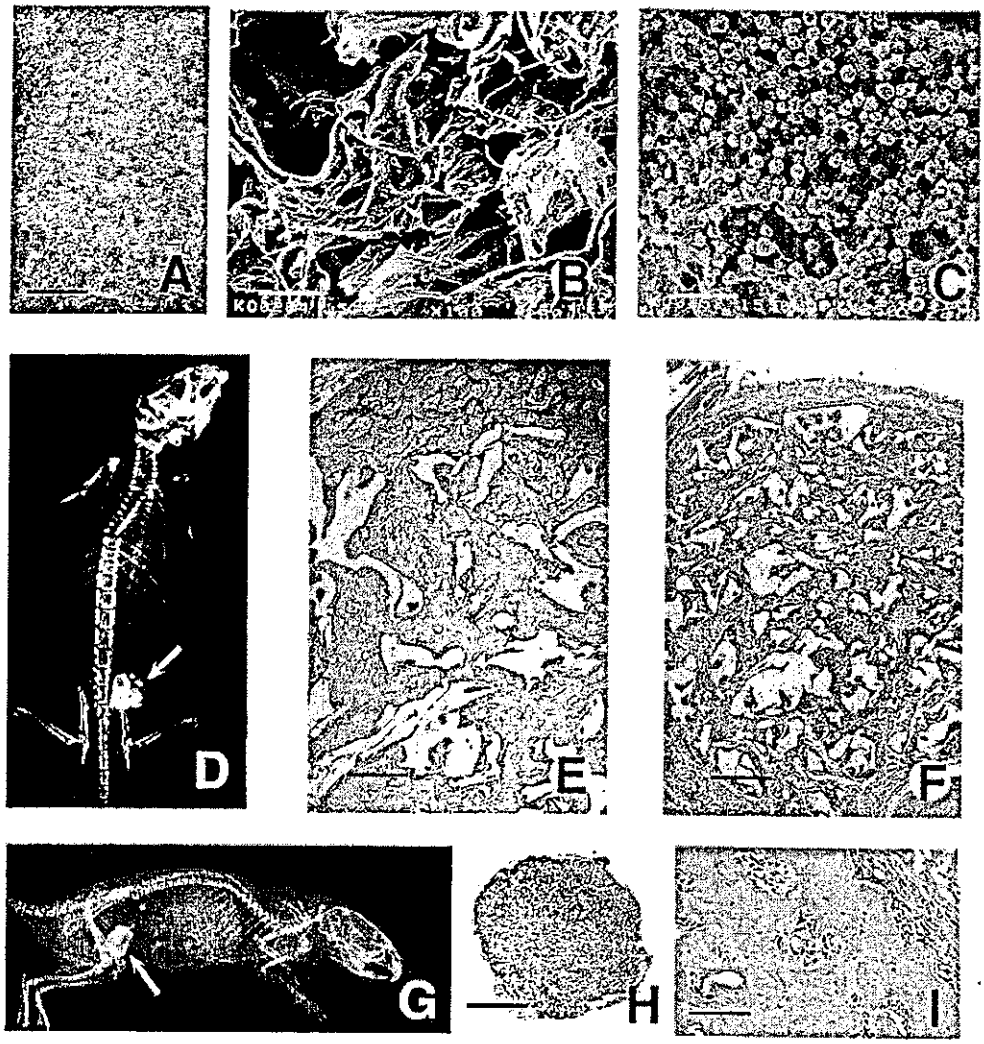

Fig. 5. Generation of custom-shaped bone by KUSA/A1 cells with PLGA-collagen sponge. PLGA-collagen sponge was used as a scaffold for KUSA/A1 cells. A: Macroscopic view of PLGA-collagen sponge. B: SEM of surface morphology of PLGA-collagen sponge without cells. C: SEM of PLGA-collagen sponge, into which KUSA/A1 cells were implanted. PLGA-collagen sponge, into which KUSA/A1 cells were implanted, was transplanted into the syngeneic mice. Six (D–F) or 11 (G–I) weeks after the transplantation, the KUSA/A1 in the sponge was excised for analysis. D: Soft X-ray of KUSA/A1 bone that is seen as an electron-dense, cuboidal bone. E: Histological examination of the bone generated by KUSA/A1 and PLGA-collagen sponge. Bone trabeculae were clearly seen. F: Histological examination of transplanted PLGA-collagen sponge without cells. Note that foreign body reaction was seen inside the transplanted sponge, but no bone was formed. G: Photo for soft X-ray of complete cuboidal bone by KUSA/A1 and PLGA-collagen sponge. H: Histology of complete cuboidal bone with high density of bone trabeculae. Note that bone was formed even in the center of the sponge. I: High power view of H. Scale bars: 350 (A), 100 (B), 72 (C), 200 μm (E and F), 5 mm (H), 250 μm (I).

Surface marker expression on KUSA/A1 cells

We analyzed the cell surface markers on the clonal KUSA/A1 cells by flow cytometry to better characterize these cells. Cells were incubated with monoclonal antibodies. Controls included cells stained with individual isotype (mouse IgG1 or rat IgG2a). Incubations were performed in the presence of mouse immunoglobulin to prevent nonspecific antibody binding. The cells were found to be strongly positive (more than tenfold greater than the isotype control) for Sca-1, CD44, Ly-6C and CD140, weakly positive for CD29, and negative for c-kit, Flk-1, CD14, CD31, CD34, CD41, CD45, CD49b, CD49d, CD54, CD90, CD102, CD105, CD106, CD144 and Ly-6G (Fig. 4). Surface marker analysis of KUSA/A1 indicated that cells with strong in vivo osteogenic activity exhibited markers for osteocytes (Horowitz et al., 1994; Hughes et al., 1994; Chen et al., 1999); CD44 and Ly-6C. Interestingly, the lack of CD90 expression supported the assumption that these cells maintain a matured phenotype even when they proliferate, since CD90 is detected in the early stage of osteoblast differentiation and declines as osteoblasts mature into osteocytes. Therefore, KUSA/A1 cells, which have strong in vivo osteogenic activity, express markers for mature osteoblasts or osteocytes. This was

confirmed with the cytochemical analysis of KUSA/A1 cells in vivo and in vitro. High ALP activity, calcium deposition, and osteocalcin release with low PTH responsibility also indicate that KUSA/A1 cells are mature osteoblasts or osteocytes.

Generating custom-shaped bones derived from KUSA/A1 cells in vivo with PLGA-collagen sponge

We attempted to generate bone tissue of desired sizes and shapes with a view to future clinical applications (Fig. 5). We previously developed a PLGA-collagen sponge with a pore of specific size for this purpose (Chen et al., 2000a,b). Collagen gels suspended with KUSA/A1 cells were injected into a cube of PLGA-collagen sponge, which was then transplanted into subcutaneous tissue and the abdominal cavity. Two weeks after the transplantation, cuboidal bone was seen on X-ray and formation of bone trabeculae was confirmed histologically. This bone formation was evident at 6 weeks. At 11 weeks, the PLGA sponge turned completely to bone. Hematopoietic cells and tartaric acid-resistant acid phosphatase-positive osteoclasts were also observed in the generated bone. Control PLGA sponges without any cells transplanted into both subcutaneous tissue and abdominal cavity showed no evidence of calcification or ossification.

DISCUSSION

Establishment of rapid and efficient membranous osteogenesis system by mature osteoblasts

We have devised and tested a system for regeneration of bone of specific shape and size that can be successfully implanted into mice using a clonal mature osteoblast cell line. These cells consistently differentiate to form bone both in vitro and in vivo. Very rapid, within 2 days, in vivo bone formation was obtained using mature KUSA/A1 osteoblasts that were cultured for 5–7 days at confluence before implantation. Cells implanted at semiconfluence, in contrast, took 2 weeks to generate bone (Umezawa et al., 1992). Since osteoprogenitor cells divide early in culture and have a limited capacity for self-renewal (McCulloch et al., 1991), these results suggest that use of mature osteoblasts is prerequisite for rapid bone formation in vivo. The in vivo osteogenic activity of the KUSA/A1 cells seems to be at least mediated by humoral factors produced by the cells per se, since bone formed not only inside the diffusion chamber but also outside the chamber, when the cells were transplanted into the chamber.

It would be interesting to assess the possibility of using mature osteoblasts as a therapeutic agent. Injection of isolated mature osteoblasts into a bone defect or fracture site would be more efficient means of accelerating bone fusion with minimal invasion than injection of all types of marrow cells into fracture sites.

PLGA-collagen sponge gave control over the shape and size of osteogenesis

We hypothesized that provision of a scaffold may effectively limit and guide new bone formation. Inoculation of KUSA/A1 cells cultured in collagen gel successfully induced bone in vivo, but collagen gel is too soft to maintain any specific desired shape in vivo, especially at the site of bone defects. However, the PLGA-collagen sponge could both support osteoblast-mediated bone formation and retain its shape in vivo. PLGA-collagen sponge has a number of advantages as a scaffold for bone regeneration: (a) it is easily shaped; (b) the time course of degradation, mechanical strength, and plasticity can be easily modulated by changing the ratio of PLA and PGA; (c) it has a high affinity for cells, mediated by collagen; (d) cells can be injected evenly throughout the sponge so that the sponge turns completely to bone.

In regenerating articular cartilage, it is an important issue how to make mechanically strong, fully weight-bearing cartilage. To this end, subchondral bone, which undercoats cartilage and confers mechanical strength to articular cartilage needs to be generated. Cartilage has been successfully regenerated, however, connection between bone and regenerated cartilage was incomplete. Our present studies suggest that osteoblasts in a scaffold serve a possible candidate for a glue to bind subchondral bone and regenerated cartilage.

Importantly, custom-shaped bone regeneration using mature osteoblasts proved to be successful. Furthermore, transplanted KUSA/A1 cells did not transform into malignant cells, form any abnormal extracellular matrices, or induce any significant inflammatory reactions. The next step will be to isolate human counterparts to these mouse cell lines. Cell separation of osteoblasts from human marrow stroma and inoculation of the cells with appropriate scaffold will provide us a new ways of osteogenesis engineering.

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Hydrostatic Fluid Pressure Enhances Matrix Synthesis and Accumulation by Bovine Chondrocytes in Three-Dimensional Culture

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Monolayer cell cultures and cartilage tissue fragments have been used to examine the effects of hydrostatic fluid pressure (HFP) on the anabolic and catabolic functions of chondrocytes. In this study, bovine articular chondrocytes (bACs) were grown in porous three-dimensional (3-D) collagen sponges, to which constant or cyclic (0.015 Hz) HFP was applied at 2.8 MPa for up to 15 days. The effects of HFP were evaluated histologically, immunohistochemically, and by quantitative biochemical measures. Metachromatic matrix accumulated around the cells within the collagen sponges during the culture period. There was intense intracellular, pericellular, and extracellular immunoreactivity for collagen type II throughout the sponges in all groups. The incorporation of [^{35}S]-sulfate into glycosaminoglycans (GAGs) was 1.3-fold greater with constant HFP and 1.4-fold greater with cyclic HFP than in the control at day 5 ($P < 0.05$). At day 15, the accumulation of sulfated-GAG was 3.1-fold greater with constant HFP and 2.7-fold with cyclic HFP than the control (0.01). Quantitative immunochemical analysis of the matrix showed significantly greater accumulation of chondroitin 4-sulfate proteoglycan (C 4-S PG), keratan sulfate proteoglycan (KS PG), and chondroitin proteoglycan (chondroitin PG) than the control ($P < 0.01$). With this novel HFP culture system, 2.8 MPa HFP stimulated synthesis of cartilage-specific matrix components in chondrocyte cultured in porous 3-D collagen sponges. J. Cell. Physiol. 193: 319–327, 2002.

Various physical and/or physicochemical factors influence the development, growth, and metabolic function of cartilage (Mow et al., 1992; Urban, 1994). Compressive dynamic loading of cartilage disks introduces deformations and other changes within the tissue, such as hydrostatic pressure, interstitial fluid flow, and streaming potential (Ratcliffe and Mow, 1991). It has been shown in vitro that externally applied hydrostatic pressure, oxygen tension, and interstitially generated osmotic and swelling pressures influence the metabolic function of cartilage disks (Lippiello et al., 1985).

Three-dimensional (3-D) agarose culture systems have been used as models for the effects of physical stimuli on chondrocytes (Buschmann et al., 1995; Lee and Bader, 1997). Synthetic responses to hydrostatic compression of 3-D agarose chondrocyte cultures depends upon the length of time the cells are maintained in culture. Buschmann et al. (1995) suggested that cell–matrix interactions and extracellular physicochemical effects are more important stimuli for matrix synthesis than are matrix-independent cell deformation under static compression. Therefore, a culture system is needed that allows for the investigation of pericellular accumulation of extracellular matrix (ECM) in 3-D tissue morphology.

We previously reported a method of producing 3-D collagen sponges that supports the viability and activity of cells in vitro, including maintenance of chondrocytic phenotype (Mizuno and Glowacki, 1996a,b). Recently, we showed that perfusion of medium at low flow rates (1.3 ml/min) enhances the viability and function of murine bone marrow cells (Glowacki et al., 1998) and murine osteosarcoma cells (Mueller et al., 1999) cultured

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at high density in 3-D collagen sponges. On the basis of those data, we designed a novel pressure/perfusion culture system to apply hydrostatic fluid pressure (HFP) to the medium perfusing the sponges. With this system, we tested the hypothesis that HFP at physiological magnitude stimulates chondrogenesis in culture. The magnitude of pressure applied was 2.8 MPa, which was within the physiological range of 0–3.5 MPa that is achieved during normal walking (Hodge et al., 1986). We measured the effects of HFP on accumulation of ECM and on incorporation of sulfate into glycosaminoglycans (GAGs) in bovine articular chondrocytes (bACs) in 3-D collagen sponges.

MATERIALS AND METHODS

Fabrication of porous collagen sponge

Porous collagen sponges were made as previously reported (Mizuno and Glowacki, 1996b). In brief, a solution of 0.5% pepsin-digested collagen from bovine skin (Cellagen™ ICN Biomedical, Costa Mesa, CA) was neutralized with HEPES and $NaHCO_3$; 250 µl of this collagen solution was poured into a mold and frozen at −20°C. Moist tissue paper was placed over the surface of the frozen collagen to prevent the formation of an impenetrable layer during lyophilization. After lyophilization, the paper was removed and each side of the collagen sponge was irradiated by UV light for 3 h at a distance of 30 cm. The final dimensions of the sponge were 7 mm in diameter and 1.5 mm thick.

Isolation of bACs

bACs were prepared from shoulders of 2–3-week-old calves obtained from a local abattoir within 3 h of slaughter (Mizuno and Glowacki, 1996a). The joints were exposed under aseptic conditions, and the cartilage was sliced (< 1 mm thick) with a surgical blade. The slices were minced into flakes of about 1 mm^3, rinsed with cold phosphate-buffered saline (PBS) three times, and digested with a solution of 0.15% collagenase (CLS 1, Worthington Biochemical, Freefold, NJ) in Ham's F12 medium (GIBCO/BRL Laboratory, Grand Island, NY) with gentle shaking overnight at 37°C. The released cells were rinsed with PBS three times. Cells were suspended in Ham's F12 medium containing 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 100 U/ml penicillin, and 100 µg/ml streptomycin (GIBCO/BRL Laboratory).

Cell seeding and pre-culture

A cell-seeding chamber (Fig. 1a) was used for seeding and initial (3-day) incubation of bACs in the collagen sponges (Mizuno and Glowacki, 1996a). The seeding chamber was used only for cell seeding. This chamber allows for easy handling. A 50-µl aliquot containing 3×10^6 cells was injected into each collagen sponge with a 25-gauge needle. Devices were placed in deep 6-well culture plates (3.5 mm diameter and 10 mm depth, Falcon B-D™, Becton Dickinson, Franklin Lakes, NJ.) containing 5 ml of medium per well to facilitate diffusion of the medium. Cultures were pre-incubated for 3 days in a humidified atmosphere of 5% CO_2 in air at 37°C. For each set of experiments, the cells in the control and in the treated sponges were isolated from the same animal.

Pressure/perfusion culture system

A pressurized and perfused culture system was designed for the application of positive HFP to the sponges with constant perfusion of the medium (Fig. 1b). The system was composed of a medium reservoir, a debubbler™ (Scientific Systems, State College, PA), a single-piston cylinder pump (ACUFLOW Series 1™ for HPLC, Scientific Systems), a pulse damper (Takagi Industry, Shizuoka, Japan) with a pressure gauge (AP-15 and AP-80A, Keyence, Japan), a glass column (a borosilicate glass column, 1 cm in diameter and 10 cm in length: AP-1, Waters, Franklin, MA), and a back-pressure regulator (Upchurch, Oak Harbor, WA) or a computer-assisted back-pressure control device (Takagi Industry). For cyclic pressure, the back-pressure was regulated using a needle valve and a spring. A computer-controlled actuator and step motor changed spring tension at a set magnitude and duration. The components exposed to the medium, such as tubing, ferrules, and connectors, were made of chemically inert polyetheretherketone or ethylene-tetrafluoroethylene (Tefzel™). All system components were either autoclaved before each experiment or sterilized by soaking in 70% ethanol. All components of the apparatus except for the pump, a debubbler, and a pulse damper/pressure sensor, were maintained within an incubator. The distance between the pulse damper and the column inlet was 1.5 m, appropriate to maintain the medium temperature at 37°C. The debubbler, the pump head, and the pulse damper were wrapped with heat-exchange tubing circulated with water at 37°C to maintain the inlet medium at 37°C. Under these experimental conditions, medium temperature was validated (data not shown) and perfusion rate was validated. Under variable pressure/perfusion conditions, the perfusion flow rate was 0.33 ± 0.015 ml/min.

After 3 days of preincubation, the sponges were removed from the cell-seeding chambers, randomly divided into test groups, and transferred to the pressure-proof column. The HFP was the same value within a sponge at all points in the column. HFP in the system was validated.

Test modes were constant perfusion (0 MPa), cyclic (0.015 Hz) 2.8 MPa (Fig. 1c), and constant 2.8 MPa HFP (Fig. 1d). In all experimental cultures, medium was perfused at a rate of 0.33 ml/min. A set of 24 sponges was suspended in a column with a medium volume of 10 ml/sponge in the system. The medium was changed every 5 days. After 5 and 15 days of experimental conditions, three sponges were harvested for histological analysis and six sponges were harvested for biochemical assays. Six sponges were incubated with 5 µCi/ml [^{35}S]-sulfate for measurement of sulfate incorporation into GAGs at day 5. For the biochemical assays, sponges were frozen at −70°C until all samples were harvested.

Histological evaluation

Triplicate specimens from each time point were fixed overnight in 2% paraformaldehyde, 0.1 M cacodylic buffer (pH 7.4), for microscopic evaluation. One sponge from each group was embedded in glycolmethacrylate (JB-4, Polysciences, Warrington, PA), and 10 µm sections were stained with 0.2% toluidine blue O (Fisher Scientific, Pittsburgh, PA) at pH 4.0. The other sponges

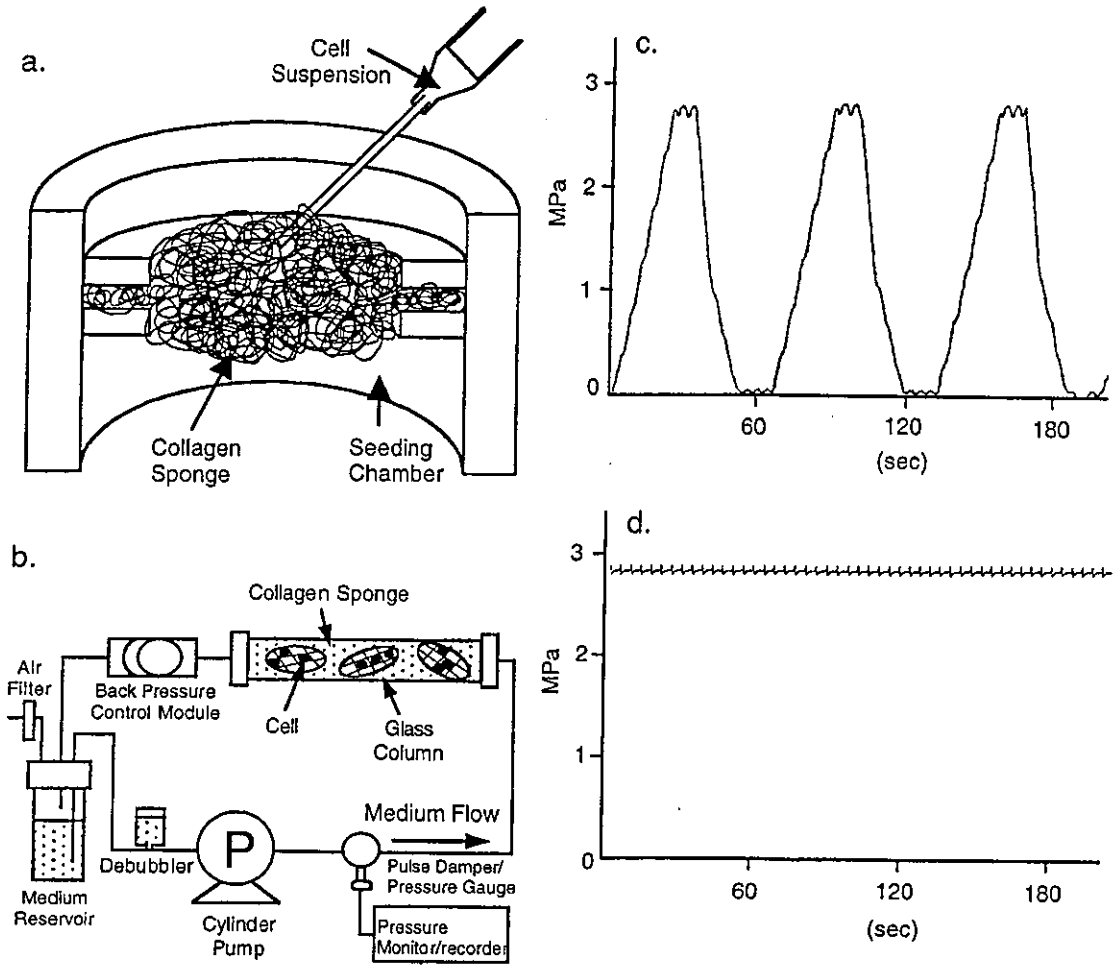

Fig. 1. a: Representation of a porous three-dimensional (3-D) collagen sponge contained within a seeding chamber. A suspension of chondrocytes was injected with a 25-gauge needle. b: The 3-D culture system used to apply medium perfusion and hydrostatic fluid pressure (HFP) to collagen sponges suspended in a glass column. The system is composed of a medium reservoir with an air filter, a debubbler, a cylinder pump to circulate medium at a precise flow rate, a pulse damper/pressure gauge to reduce fluctuation by pump stroke and synchronized vibration by pump motor, a glass column, and a back-pressure control module that regulates HFP at either a fixed pressure level or programmed cyclic pressure. Sponges were freely suspended in medium in the column. The pump head, the pulse damper, the debubbler, and tubing were wrapped in a water jacket to maintain constant temperature. c: Recording of pressure achieved with settings for 2.8 MPa, cyclic, 0.015 Hz, and 0.33 ml/min. d: Recording of pressure achieved with settings for 2.8 MPa constant and 0.33 ml/min.

were embedded in paraffin and sectioned (10 μm) for immunohistological evaluation. Before fixation, a sponge was cut into two pieces and stained routinely with fluorescent dye (Live/Dead® viability/cytotoxicity kit, Molecular Probes, Eugene, OR) followed according to manufacturer's instruction to validate cell viability.

Immunohistochemistry for collagen type II

Paraffin-embedded sections were dewaxed with xylene and a series of graded ethanol solutions. They were blocked with 3% normal horse serum in a humidified chamber for 20 min at room temperature. The sections were then incubated with rabbit anti-bovine collagen type II antibody (Chemicon, Temecula, CA) diluted 1:100 in PBS for 1 h at room temperature. After three rinses with PBS, the sections were incubated with biotinylated goat anti-rabbit IgG according to the manufacturer's instructions (Vectastatin™ABC, Vector Laboratory, Burlingame, CA). Color was developed with 3,3'-diaminobenzidine (DAB) and nickel according to the manufacturer's instructions in a DAB kit with nickel (Vector Laboratory).

Measurement of [^{35}S]-sulfate incorporation into GAGs

Twenty-five microCuries of [^{35}S] sodium sulfate (NEN Life Science Products, Boston, MA) in 5 ml of medium per sponge was added during the last 18 h of the 5-day culture period. For technical safety in experiments with hydrostatic pressure, 3.5 ml/sponge of medium was left to maintain the running system, and 1.5 ml/sponge of fresh medium containing [^{35}S] was added to the medium reservoir. Each sponge was digested with 1 ml of 125 μg/ml papain (Sigma, St. Louis,

MO) in 5 mM cysteine–HCl, 0.05 M EDTA, and 0.1 M sodium phosphate for 16 h at 60°C (Kim et al., 1994). The samples were heat-treated for 45 min at 95°C, and 0.5 ml of each sample was collected after removal of free [^{35}S] by Sephadex G-25 chromatography (PD-10, Amersham Pharmacia Biotech, Piscataway, NJ). [^{35}S] was measured in a scintillation counter (Packard Instrument Company, Meriden, CT) with scintillation fluid (Scintiverse^{TM}, Fisher).

Fluorimetic assay for DNA content

The DNA content of each sponge was measured by the fluorescent dye method (Kim et al., 1988; Rymaszewski et al., 1990). Papain-digested samples for the [^{35}S]-sulfate assay were prepared for DNA measurement without heat treatment. Ten-microliter samples were added to a solution of 2 ml Hoechst 33258 (4.0 μg/ml, Polysciences), 0.1 M NaCl, 10 mM Tris-HCl, and 1 mM EDTA (pH 7.4). The mixture was measured with a fluorometer (TKO 100, Hoefer, San Francisco, CA) using calf thymus DNA as the standard (Clontech Laboratory, Palo Alto, CA).

Biochemical extraction for dimethylmethylene blue (DMB) assay and proteoglycan analysis

For biochemical measurements, frozen sponges were minced into 1-mm^3 pieces with a surgical blade. Each of six replicate samples was extracted for 48 h at 4°C in 1 ml of 4 M guanidine-hydrochloride, 10 mM EDTA (pH 5.8), with protease inhibitors (0.1 M e-aminohexanoic acid and 0.005 M benzamidine hydrochloride) (Rennard et al., 1981). After centrifugation at 3,000*g* for 5 min, supernatants were precipitated with 3 × volume of 1.3% potassium acetate in absolute ethanol. The ethanol precipitate was repeated twice, and the final precipitation was used for measurement of S-GAG (Farndale et al., 1982), chondroitin 4-sulfate proteoglycan (C 4-S PG; Couchman et al., 1984), chondroitin proteoglycan (chondroitin PG; Couchman et al., 1984), and keratan sulfate proteoglycan (KS PG; Caterson et al., 1983; Sorrell et al., 1988).

Colorimetric assay for sulfated glycosaminoglycan (S-GAG content or DMB assay)

Two milligrams of sodium formate (Sigma) was dissolved in 2 ml of formic acid (Sigma) and mixed with 900 ml of deionized water. Sixteen milligrams of 1,9-DMB (Aldrich, Milwaukee, WI), dissolved in 5 ml of absolute ethanol, was added to the formate solution and brought to a final volume of 1,000 ml with deionized water (Farndale et al., 1982). The ethanol precipitate was resuspended with deionized water. Thirty microliters of each sample was added to 150 μl of the DMB solution in a 96-well titer plate (Falcon). The optical density of the sample was measured at 540–595 nm with a microtiter plate reader (550, Bio-Rad, Cambridge, MA). The GAG concentration was determined from standard curves for shark chondroitin sulfate (Sigma).

ELISA for measurement of chondroitin 4-sulfate, chondroitin, and KS PG

As an index of the differentiated function of chondrocytes, accumulation of C 4-S PG (Couchman et al., 1984), chondroitin PG (Couchman et al., 1984), and KS PG (Caterson et al., 1983) was measured immunochemically as previously reported (Mizuno and Glowacki, 1996a). The ethanol precipitates were dissolved in carbonate buffer (35 mM $NaHCO_3$, 18 mM Na_2CO_3, pH 9.8) and re-precipitated in the same manner. Dilutions of the samples were subjected to immunochemical analysis (Farndale et al., 1982). A proteoglycan monomer from bovine nasal cartilage (ICN Biomedicals, Costa Mesa, CA) was used as a standard. Fifty-microliter aliquots of each sample or standard were coated onto 96-well plates overnight at 4°C, rinsed, and digested for 1 h at 37°C with 50 μl of 0.1 U/ml protease-free chondroitinase ABC (Seikagaku America, Falmouth, MA) in 0.1 M Tris-HCl, and 0.03 M sodium acetate (pH 8.0). Each well was treated with 200 μl of blocking solution (BLOTTO^{TM}, Pierce, Rockford, IL). After the chondroitinase ABC digestion, antibodies to chondroitin Di-4 sulfate proteoglycan (Clone; 2-B-6, Seikagaku America), to chondroitin (Clone; 1-B-5, Seikagaku America), and to keratan sulfate (Clone; 5- D-4, Seikagaku America) were used at a 1: 3,000 dilution in PBS (pH 7.4) and incubated for 2 h at room temperature. The second antibody, goat anti-mouse IgG + IgM-biotin conjugate (Pierce), was used at a 1:20,000 dilution in PBS for a 1 h incubation. For enhancement, phosphatase–streptavidin conjugate (GIBCO/BRL Laboratory) was added at 1:1,000 dilution with PBS for 1 h. Between steps, the wells were rinsed with 0.05% Tween 20-PBS. Each well was incubated with 100 μl of 4 mg/ml *p*-nitrophenylphosphate (GIBCO/BRL Laboratory) in a buffer containing 22 mM sodium carbonate, 28 mM sodium bicarbonate, and 1 mM $MgCl_2$ (pH 9.8) for 1 h. One hundred microliters of 1 N NaOH was added to terminate each reaction. The optical density at 405 nm was measured with a microtiter plate reader (Bio-Rad).

Data analysis

Quantities of S-GAGs and C 4-S PG, chondroitin PG, and KS PG were expressed per sponge, and comparisons were made by a one-way Student's *t*-test for statistical significance. Experiments were repeated three times to confirm the reproducibility of the results.

RESULTS

Cellularity, shape, and accumulation of ECM in 3-D collagen sponges

Chondrocytes were distributed throughout the sponge with some cellular aggregation and accumulation along collagen fibers after 3-days of preincubation in conventional culture dishes (Fig. 2a). There were deposits of metachromatic ECM throughout the collagen framework. After 15 days with perfusion but without HFP, abundant metachromatic matrix composed of fine fibrils and homogenous deposits was visible (Fig. 2b). Matrix accumulation was greatest in areas of high cell density. Many cells were enclosed in lacunae. Sponges that were exposed to cyclic 2.8 MPa appeared to have accumulated a much denser metachromatic matrix (Fig. 2c). Sponges exposed to constant 2.8 MPa (Fig. 2d) accumulated more matrix than did the control sponges. Immunohistochemical staining of 15-day specimens showed intracellular localization of collagen type II immunoreactivity in the perfused sponges (Fig. 3a). Sponges exposed to cyclic

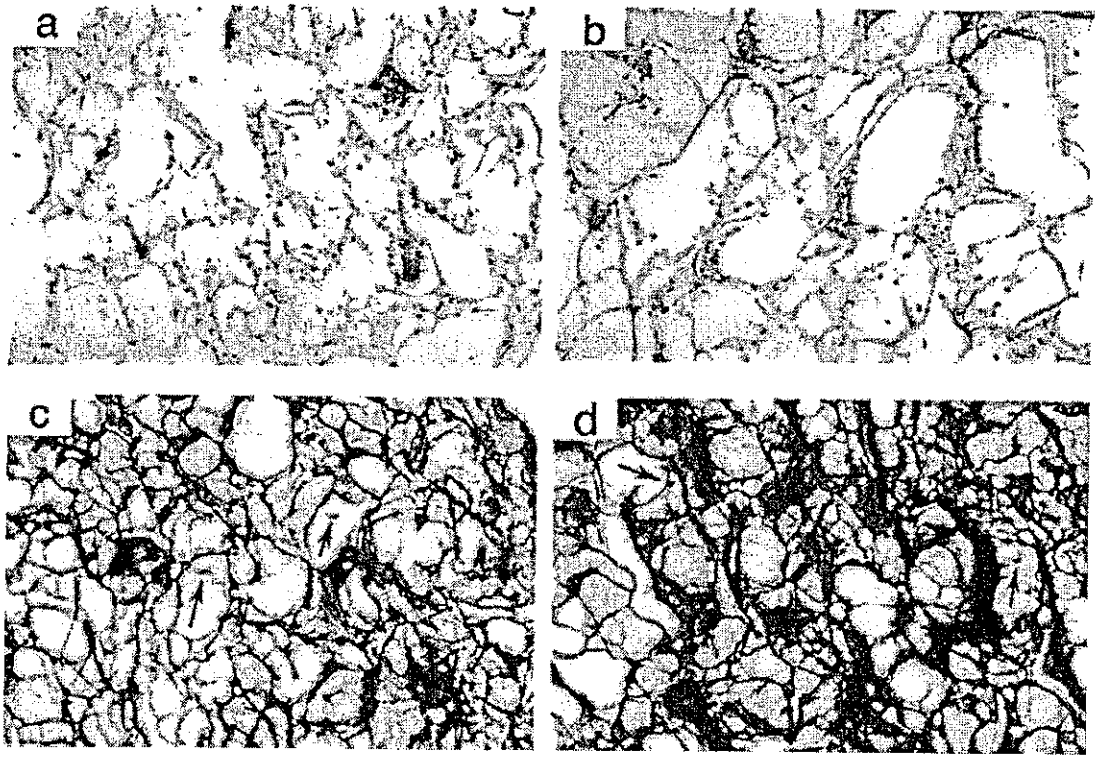

Fig. 2. Photomicrographs of thick sections of collagen sponges exposed to different treatments. a: After 3 days of preincubation, metachromatic matrix is evident around cells. In some areas, isolated cells are enclosed in lacunae. b: After 15 days without HFP (0 MPa), abundant metachromatic matrix appears as homogenous deposits. c: After 15 days of exposure to cyclic 2.8 MPa, there is dense metachromatic matrix obscuring cellular detail. d: After 15 days exposure to constant 2.8 MPa, dense matrix is evident throughout the sponge. Arrows indicate chondrocytes (× 120 magnification, Toluidine blue, 10 μm thick section).

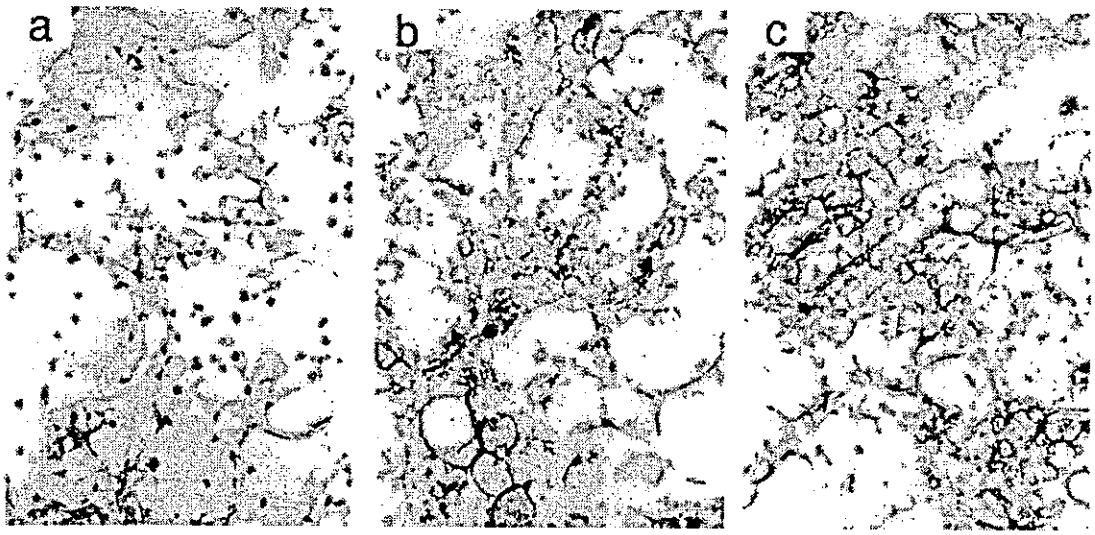

Fig. 3. Photomicrographs of collagen type II immunoreactivity in sponges with chondrocytes after 15 days of culture. a: In a sponge not exposed to HFP (0 MPa), immunoreactivity is localized to the chondrocytes. b: In a sponge exposed to cyclic 2.8 MPa, immunoreactivity is seen with intracellular, pericellular, and extracellular distribution. c: In a sponge exposed to constant 2.8 MPa, intense reactivity is seen pericellularly and extracellularly (× 120 magnification, 10 μm thick section).

2.8 MPa showed intracellular, pericellular, and extracellular immunoreactivity to collagen type II (Fig. 3b). With constant 2.8 MPa, there were more intense pericellular deposits (Fig. 3c).

Incorporation of [^{35}S]-sulfate into GAGs

Six sponges from each culture condition were harvested for biochemical evaluation after 5 days of exposure to experimental conditions. The average DNA content of each group was 84 μg/sponge, with no significant differences among the groups. The amount of [^{35}S]-sulfate incorporated in the sponges exposed to cyclic 2.8 MPa (456 ± 61 cpm/μg DNA) was 1.4-fold higher than in the control (324 ± 38, $P < 0.05$) and [^{35}S]-sulfate incorporation in those exposed constant 2.8 MPa (411 ± 96) was 1.3-fold higher than in the control ($P < 0.01$) (Fig. 4).

Accumulation of ECM by chondrocytes

ECM was extracted from the collagen sponges and measured biochemically and immunochemically. Total sulfated GAG (S-GAG) content was measured by DMB assay after 0, 5, and 15 days of treatment. In the control group (0 MPa), S-GAG content increased twofold between day 0 (19.7 ± 7.1 μg/sponge) and day 5 (37.5 ± 7.1, $P < 0.01$). The amount of S-GAG was sustained (36.8 ± 5.5 μg/sponge) at day 15 (Fig. 5a). After 5 days, the S-GAG content in sponges exposed to cyclic (77.2 ± 14.5 μg/sponge) and constant (68.7 ± 12.3) 2.8 MPa pressure was 2- and 1.8-fold greater than that in the control, respectively ($P < 0.01$). After 15 days, the S-GAG content in sponges that were exposed to cyclic (99.4 ± 19.9 μg/sponge, $P < 0.01$) and constant (114.1 ± 8.5) 2.8 MPa was 2.7- and 3.1-fold greater than that in the control, respectively ($P < 0.01$). S-GAG levels in groups exposed to cyclic or constant 2.8 MPa pressure were not significantly different at either day 5 or 15.

The amount of C 4-S PG in control sponges (0 MPa) was similar on day 0 (19.5 ± 7.2 μg/sponge) and day 5 (19.5 ± 3.6), and was significantly decreased on day 15 (13.5 ± 3.0, $P < 0.02$) (Fig. 5b). On day 5, the C 4-S PG level in sponges exposed to cyclic (48.8 ± 14.1 μg/sponge) and constant (38.4 ± 14.7) 2.8 MPa pressure was 2.5- ($P < 0.01$) and 2.0-fold ($P < 0.05$) greater, respectively, than that in the control (19.5 ± 3.6). The C 4-S PG content in both treatment groups was similar on day 5 and day 15.

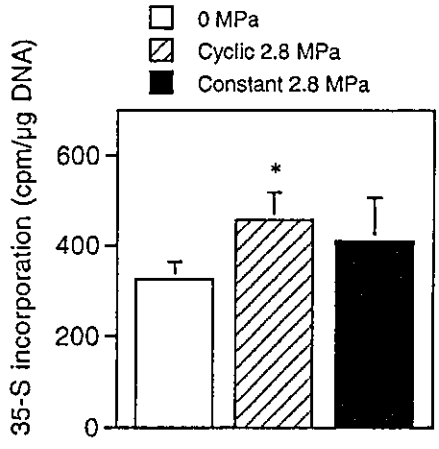

Fig. 4. [^{35}S]-sulfate incorporation (cpm per μg DNA) into GAG by bovine articular chondrocytes (bACs) in collagen sponges exposed to different culture conditions for 5 days. (Bars represent the means of five or six samples ± standard deviation. *$P < 0.05$; **$P < 0.01$).

The amount of chondroitin PG in the control group was similar on day 0 (13.2 ± 5.0 μg/sponge) and day 5 (15.7 ± 4.1) and increased 3.4-fold from day 5 to day 15 (52.7 ± 17.8, $P < 0.01$) (Fig. 5c). After 5 days, chondroitin PG in sponges exposed to cyclic and constant (38.6 ± 6.4 μg/sponge) 2.8 MPa was 2.2- (35.1 ± 8.8, $P < 0.01$) and 2.5-fold ($P < 0.01$) greater than in the control, respectively. After 15 days, chondroitin PG in sponges exposed to cyclic (100.4 ± 21.9 μg/sponge) and constant (105.3 ± 33.8) 2.8 MPa was 1.9- ($P < 0.01$) and 2-fold ($P < 0.01$), respectively, greater than in the control. Between days 5 and 15, chondroitin PG in sponges exposed to cyclic and constant 2.8 MPa increased 2.7- ($P < 0.01$) and 2.8-fold ($P < 0.01$), respectively.

Content of KS PG in the control increased significantly between day 0 (3.30 ± 0.84 μg/sponge) and day 5 (5.02 ± 0.69, $P < 0.01$). Thereafter (Fig. 5d), the amount of KS PG in the control decreased significantly (2.59 ± 0.85, $P < 0.01$). KS PG in sponges exposed to cyclic (6.23 ± 1.35 μg/sponge) and constant (5.02 ± 1.22) 2.8 MPa increased 1.9- ($P < 0.01$) and 1.5-fold ($P < 0.05$), respectively, between days 0 and 5. At day 15, the KS PG level in sponges exposed to cyclic (6.93 ± 0.63 μg/sponge) and constant (6.96 ± 1.30) 2.8 MPa was sustained at a significantly greater level than in the control (2.59 ± 0.85), by 2.7- ($P < 0.01$) and 2.7-fold ($P < 0.01$), respectively.

DISCUSSION

These studies show that HFP at a physiologically relevant level stimulates chondrogenesis by bACs in a 3-D culture system. This report addresses the effects of medium HFP without inducing significant cellular deformation. This is distinct from cellular effects of mechanical loading. bACs were cultured in porous 3-D collagen sponges to which cyclic (0.015 Hz) or constant HFP was applied at 2.8 MPa for up to 15 days. Metachromatic matrix accumulated around the cells within the collagen sponges over time. Cyclic and constant 2.8 MPa HFP stimulated synthesis of S-GAG and accumulation of S-GAG, C 4-S PG, chondroitin PG, and KS PG.

In articular cartilage, chondrocytes are embedded in a highly hydrated ECM composed of sulfated proteoglycans that are conjugated to hyaluronan and associated with fibers of collagen type II. In that environment, the cells are exposed to various interstitial stresses (e.g., osmotic pressure and swelling pressure) and external stress (e.g., hydrostatic pressure). In addition, HFP and osmotic pressure induced by bound water and proteoglycan differ from HFP of unbound water based on the biphasic theory (Mow et al., 1980, 1999). In order to approximate in vivo conditions in studies with isolated cells, investigators have used high-density aggregates (Smith et al., 1996), suspension cultures in amorphous carriers (Benya and Shaffer, 1982; Bujia et al., 1994),

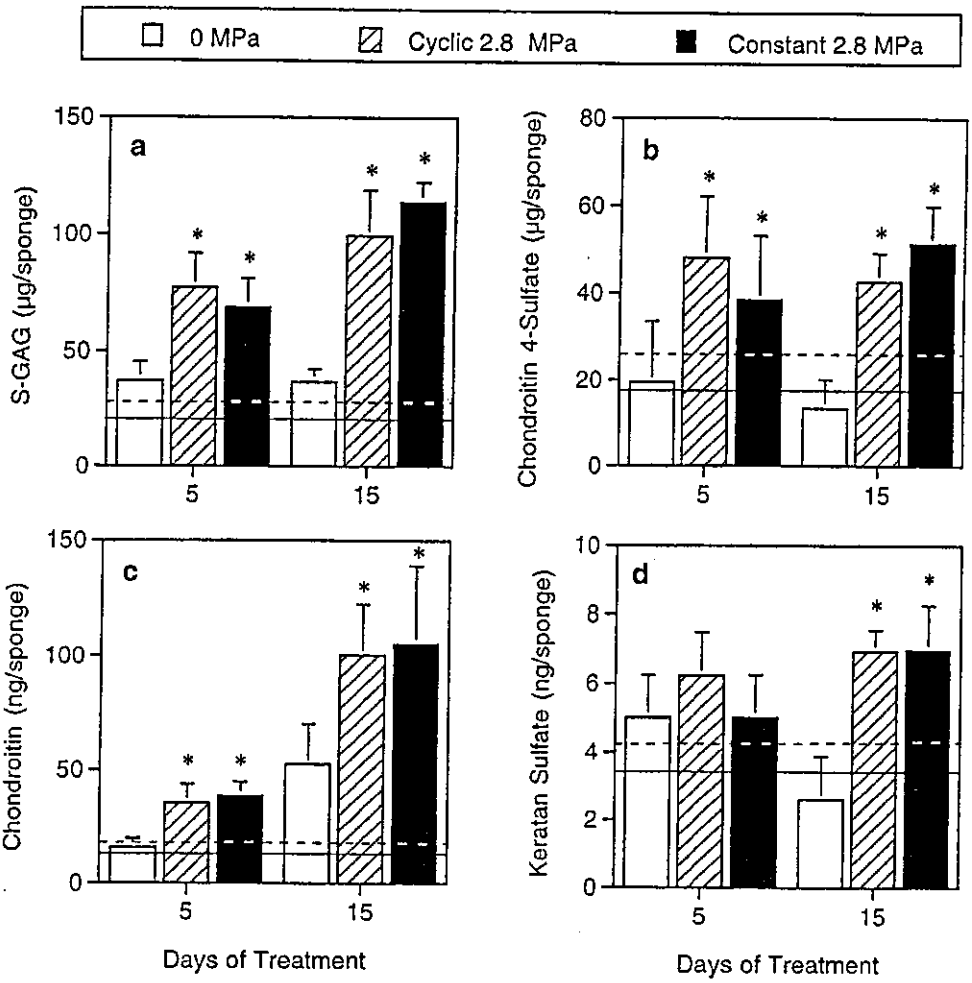

Fig. 5. Effects of culture conditions on matrix components synthesized by bACs cultured within collagen sponges for 5 and 15 days. **a**: Accumulation of sulfated glycosaminoglycan. **b**: Accumulation of chondroitin 4-sulfate proteoglycan (C 4-S PG). **c**: Accumulation of chondroitin proteoglycan (chondroitin PG). **d**: Accumulation of keratan sulfate proteoglycan (KS PG). (Solid line represents the mean value at day 0 and broken line represents the standard deviation at day 0. Bars represent the mean of five or six samples ± standard deviation. '*' indicates significant difference relative to 0 MPa at each time point, $P < 0.01$).

or 3-D scaffolds (Sittinger et al., 1994). We previously designed a porous 3-D collagen sponge as a scaffold for the characterization of cell differentiation, histogenesis, and ECM accumulation in vitro (Mizuno and Glowacki, 1996a,b; Glowacki et al., 1998) and in vivo (Gerstenfeld et al., 1996). In this study, metachromatic ECM accumulates within all treatment groups. Thus, the collagen fiber network serves as hospitable scaffolding for ECM accumulation. Moreover, this perfusion culture system was designed to provide perfusion of medium through a culture column and was shown to support the viability and function of marrow cells (Glowacki et al., 1998) of osteoblasts (Mueller et al., 1999) of oral mucosa (Navarro et al., 2001), but not of chondrocytes (Mizuno et al., 2001) in porous 3-D sponges.

The effects of mechanical stimuli on cartilage and chondrocytes have been tested with custom-designed apparatus in confined and unconfined models reviewed by Mow et al. (1999) and Mizuno et al. (1998). Confined and unconfined models include structural deformation, which may significantly change cell shape (Guilak et al., 1995; Guilak, 2000). The solid matrix of articular cartilage is incompressible when subjected to hydrostatic pressure up to 12 MPa (Bachrach et al., 1998). Biosynthetic responses to dynamic or cyclic compression, however, may be either stimulated or inhibited depending on the frequency and the amplitude of loading (Palmoski and Brandt, 1984; Klein-Nulend et al., 1987; Gray et al., 1988; Sah et al., 1989; Ostendorf et al., 1994; Buschmann et al., 1996; Torzilli et al., 1997; Mankin and Zaleske, 1998).

The effects of HFP had been evaluated on cell suspensions (Hall et al., 1991), monolayer culture, cartilage disks, and 3-D culture constructs (Carver and Heath, 1998, 1999a,b). Data from experiments with different conditions of magnitude, duration, and culture method cannot be directly compared to the results obtained from the experimental setting used in this study. However,

these referenced studies concluded that HFP promotes more accumulation of ECM than ambient pressure. Those investigators concluded that cell–matrix interactions influence the effects of cyclic hydrostatic pressure on cellular function. Studies with isolated chondrocytes also showed that proteoglycan synthesis and aggrecan mRNA expression depended on the mode of pressure (Lammi et al., 1994). GAG synthesis does not directly depend on transcription and translation, according to Smith et al., who reported that constant pressure stimulated GAG synthesis by chondrocytes in high-density monolayer cultures without effects on mRNA levels of aggrecan. Those data indicate that HFP influences the type and amount of S-GAG produced.

The novel pressure/perfusion culture system that we developed allows evaluation of cellular responses to HFP without significant deformation of cells. The system described herein permits application of HFP to cells in 3-D collagen sponges suspended in culture medium with either cyclic and constant HFP from 0 to 2.8 MPa, an approximation of peak physiologic levels of 3.5 MPa hydrostatic stress achieved during normal walking (Hodge et al., 1986). In this study, chondrocytes were isolated from shoulder cartilage of 2- to 3-week-old calves with a body weight of ~40–60 kg. Based on data that show that ~60% of body weight load is placed on the front legs in a quadruped animal (McDuffee et al., 2000, Brama et al., 2001), we chose 2.8 MPa HFP as an experimental magnitude. The DMB assay for S-GAGs represents all sulfated molecules, which are abundant in cartilage. Application of HFP significantly increased the accumulation of metachromatic matrix, S-GAG, C 4-S PG, chondroitin PG, and KS PG. Chondroitin PG is more abundant than the sulfated PGs in embryonic (Mathews, 1972) and neonatal articular cartilage (Bayliss et al., 1999). With HFP treatment, the number of residues of chondroitin PG epitopes increased with time relative to the control. HFP may promote immature chondrocyte metabolism. The KS PG is a specific but minor proteoglycan of aggrecan in cartilage (Hascall et al., 1976). The increase in KS PG signifies that aggrecan accumulation was stimulated by HFP. Because the sponges were suspended in culture medium, this pressure/perfusion system does not generate HFP by contacting the sponge with a piston. HFP is transduced in all directions through the ECM, cell membrane, and intracellular organelles as a scalar unit. Although it is generally assumed that there is no deformation of fluid under hydrostatic pressure, water shrinks to a small degree even at magnitudes of physiological weight bearing (e.g., 1 L shrinks by 45 μl (0.0045%) upon application of HFP from 0.1 to 1.0 MPa) (Gevantman, 1992). Even in loading of intact cartilage, 90% of the loaded hydrostatic pressure is exerted as interstitial fluid pressure (Ateshian and Wang, 1995). Interstitial fluid flow as well as HFP need to be considered for determining the specific cellular cascade of signal transduction by pressure. This deformation-free culture system may be useful for analysis of transduction mechanisms.

The pressure/perfusion 3-D culture system used in this study is not regarded as a model for evaluation of mature cartilage tissue physiology and pathophysiology, but it has distinct advantages for evaluating the regulation of anabolic and catabolic functions of cells in a deformation-free pressure environment.

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Hybrid of Gel-Cultured Smooth Muscle Cells With PLLA Sponge as a Scaffold Towards Blood Vessel Regeneration

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Although rapid formation of a smooth inner surface is important in constructing an artificial vascular graft, a conventional model that uses a biodegradable polymer such as poly-glycolic acid needs long-term culture to form it. In another model, which uses collagen gel, it is reported that prompt formation of the smooth inner surface was achieved. But the mechanical properties were not suitable, resulting in rupture under high pressure at the arterial level. Therefore, we propose a new artificial vascular graft model made of biodegradable polymer, gel, and cells. At first we manufactured an artificial vascular graft (i.d. 5 mm, o.d.7 mm) consisting of poly-L-lactic acid (PLLA) with open pore structures by using gas-forming methods. After mixing human normal aortic smooth muscle cells (SMCs) with type I collagen solution, pores of the PLLA scaffold were filled with the mixture. The collagen mixture was made into gel in the pores of the PLLA scaffold, incubating at 37°C. WET-SEM analysis showed that the prompt formation of a smooth inner surface was achieved in the new model. The ratio of incorporation of SMCs into the artificial vascular graft became approximately 100% by using the cell–collagen mixture, whereas only 40% of SMCs were trapped in the conventional model where SMCs were inoculated as a cell–medium suspension. Therefore, it was suggested that the new artificial vascular graft model was superior in smooth inner surface formation and cell inoculation, compared with conventional models using either biodegradable polymer or gel.

Key words: Biodegradable polymer; Gel; Artificial blood vessel; Cells; Collagen

INTRODUCTION

Although there is great interest in the exploitation of novel artificial vascular grafts, development of an artificial vascular graft with a diameter smaller than 5 mm has not been successful thus far (18,20). Many kinds of artificial materials have been applied to test prosthetic vascular grafts in vivo or in vitro, but almost all materials have induced thrombogenesis on the material surface (3,4). On the other hand, because thrombogenesis does not occur in a native blood vessel in vivo, the development of an artificial vascular graft with a structure similar to that of the native blood vessel has attracted the attention of researchers in the field of vascular prosthetics.

In 1986, Weinberg and Bell first developed an artificial vascular graft composed of collagen gel, bovine aorta endothelial cells, and bovine aorta smooth muscle cells (19), but there was a drawback in that the reconstituted vascular grafts ruptured with ease under lower pressure conditions at the venous level. Niklason and Langer made an artificial vascular graft constructed from cells and a biodegradable polyester, a nonwoven poly-glycolic acid mesh, because the polyester has fine mechanical properties (12,13). Formation of a smooth surface on the artificial vascular graft was important to prevent turbulent flow, because nonphysiological flow induces arteriosclerosis in blood vessels. Therefore, a long-term 5-week culture was needed to form a smooth inner surface in their model. From the standpoint of clinical medicine, rapid formation of an artificial vascular graft with a smooth inner surface would become advantageous in use as a tissue-engineered vascular graft.

In this article our purpose is to propose a new model of an artificial vascular graft that can rapidly form a smooth inner surface. We particularly focused on developing a hybrid-type composite composed of a biodegradable scaffold and gel.

MATERIALS AND METHODS

Cell Culture

Human normal aortic smooth muscle cells (SMCs) were purchased from Clontics (MD, USA) and cultured in a basal medium based on a modified MCDB-131 sup-

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plemented with human epidermal growth factor, human basic fibroblast growth factor, gentamicin, and 5% fetal bovine serum. After reaching confluence, the SMCs were harvested with 0.05% trypsin/0.01% EDTA solution for inoculating into an artificial blood vessel.

Preparation of Artificial Vascular Graft With Small Diameter

PLLA scaffolds for artificial vascular grafts were prepared by a gas-forming method with ammonium chloride particles as a porogen and a gas-forming reagent (11). A polymer solution was prepared by dissolving PLLA in chloroform at a concentration of 83.3 mg/ml. Ammonium bicarbonate salt particulates were added to the PLLA solution (weight ratio of NH_4HCO_3 to PLLA used was 20:1) and mixed thoroughly with a spatula. The paste mixture of polymer/salt/solvent was cast into a glass mold with an inner diameter of 7 mm. After putting the PLLA paste mixture into the glass mold, a stick with diameter of 5 mm was inserted into the PLLA paste mixture. After removing the glass mold and stick from the PLLA paste mixture, the PLLA paste mixture was dried at a temperature of 60°C overnight, and immersed in an excess amount of hot water until no gas bubbles were generated.

A 0.39% collagen solution with sodium bicarbonate (3.7 g/L), 2-(4-(2-hydroxyethyl)-1-piperazinyl) ethane sulfonic acid (HEPES, 20 mM) was mixed with SMCs at a concentration of 5×10^6 cells/ml for 15 min. After inserting a glass stick, the mixture of collagen solution and SMCs was incubated to make a gel in the pores of a PLLA scaffold for 60 min. Cells not incorporated to an artificial vascular graft were collected in 1.5-ml tubes, for DNA analysis. For WET-SEM analysis, the vascular grafts were incubated for 1 day.

DNA Assay

To evaluate the inoculation efficiency of cells in an artificial vascular graft, the amount of DNA was measured by means of fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI)-fluorometry with calf thymus DNA as a standard (1,14). Cells not incorporated into the artificial vascular graft were collected in 1.5-ml tubes, washed with PBS, and homogenized. The DNA in the solution was incubated with DAPI solution, and the fluorescence was measured with a spectrophotofluorometer.

WET-SEM Analysis

After a 1-day culture of the artificial vascular graft, the samples were immersed in 4% formalin/PBS solution overnight. To visualize the inner and outer surfaces of the artificial vascular grafts, a WET-SEM (Hitachi, SM-300, Japan) was used. Under negative pressure conditions (60 Pa), the surfaces of the samples, immersed in a 4% formalin/PBS solution, were observed without any treatment.

RESULTS

To readily inoculate cells into the scaffold, development of a scaffold with an open pore structure was needed. We therefore tried to make a PLLA scaffold by using a gas-forming method with ammonium chloride particles as a porogen and a gas-forming reagent. As shown in Figure 1, development of three-dimensional scaffolds with an open pore structure for an artificial vascular graft was achieved in our experiment.

To transplant an artificial vascular graft, formation of a smooth inner surface was necessary. We therefore developed a new artificial vascular model consisting of biodegradable polymer, gel, and cell (Fig. 2). To investigate whether a smooth inner surface on an artificial vascular graft was formed or not, the surface was visualized by means of WET-SEM.

It was found that there were a lot of pores in the 200–500 μm range on the inner surface of a PLLA scaffold, as shown in Figure 3A and D. In the model of a PLLA scaffold with SMCs and collagen gel, a smooth surface made by SMCs and collagen gel was confirmed after 24 h of fabrication (Fig. 3B, E), but only a few SMCs were observed on the surface of the PLLA scaffold in a conventional model inoculating SMCs into the PLLA scaffold as cell–medium suspension.

Figure 4 shows photographs of the outsides of artificial vascular vessels. It was found that a PLLA scaffold made by using a gas-forming method had many pores on the surface (Fig. 4A, D). In our new model made by using the cell–collagen mixture, a smooth surface was formed (Fig. 4B, E). On the other hand, bare surfaces

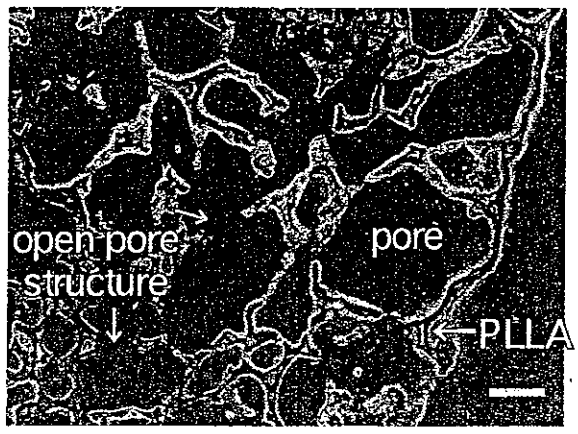

Figure 1. Cross section of a PLLA scaffold with open pore structure for artificial vascular graft. Bar: 100 μm, thickness: 8 μm.

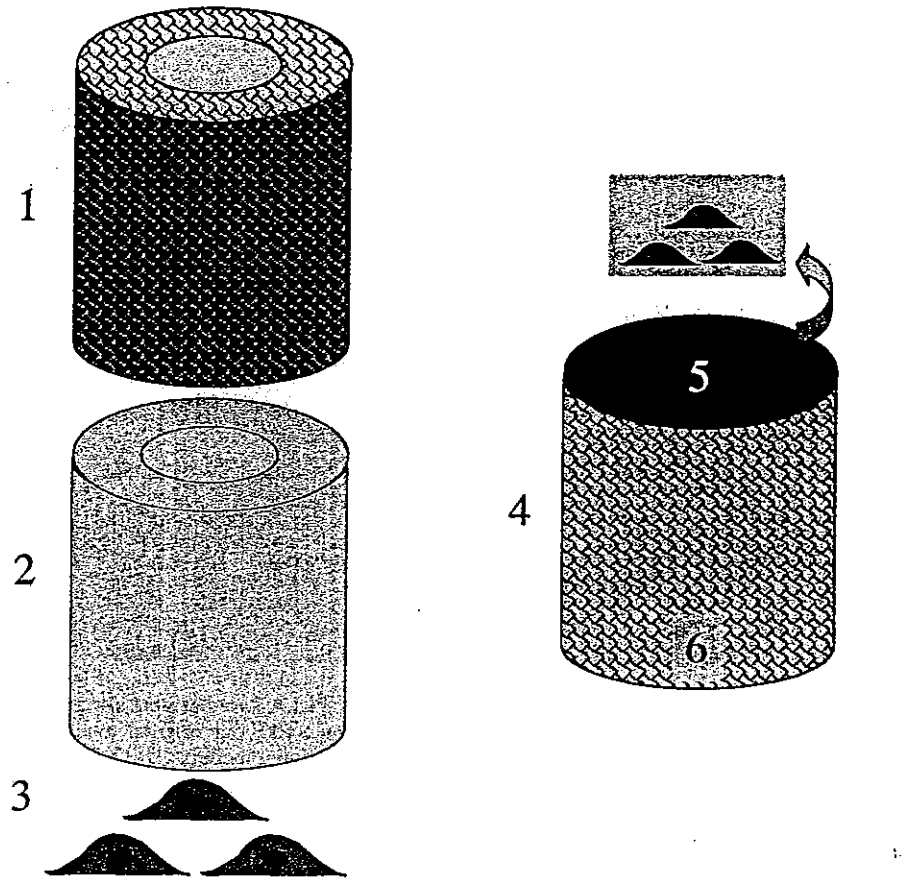

Figure 2. Schematics of our model. 1: PLLA scaffold with open pore structure. 2: Collagen solution. 3: SMCs. 4: A new artificial vascular graft model consisting of biodegradable polymer, gel, and cells. 5: Inner surface. 6: Outer surface.

were observed in a conventional model by inoculating SMCs as cell–medium suspension (Fig. 4C, F).

To verify whether SMCs could be incorporated into an artificial vascular graft, DNA amounts were quantified. The number of SMCs incorporated into the artificial vascular graft was indirectly estimated. When SMCs were inoculated into PLLA scaffolds as a collagen mixture, SMCs not incorporated into the artificial vascular grafts after gel formation were not detected (approximately 0%) according to the DNA assay as shown in Figure 5. This means that almost all SMCs inoculated into the artificial vascular grafts could be trapped by them.

When SMCs–medium suspension (control experiment) was incubated with a PLLA scaffold as a conventional model, DNA analysis showed that 60% of SMCs inoculated into the PLLA scaffold were not incorporated into the artificial vascular graft (Fig. 5). This suggests that only 40% of SMCs adhered to and were incorporated into the PLLA scaffold. It was therefore found that our new model, a combination of collagen gel and PLLA scaffolds, was a novel one, able to effectively incorporate SMCs into a three-dimensional scaffold.

DISCUSSION

In our experiments, rapid formation of a smooth inner surface was possible by combining a PLLA sponge and collagen gel. When only biodegradable scaffolds were used to make an artificial vascular graft, long-term culture of cells in the scaffolds was needed (16,17). Considering the clinical medicine and industrial aspects, rapid formation of a smooth inner surface is important. If it takes a long time to make the smooth inner surface in the artificial vascular graft, considerable expense would be required and the costs for medical treatment and health care would greatly increase. Furthermore, the risk of contamination of the culture medium by bacteria, mold, or yeast, etc., would increase. Therefore, it is thought that our new model incorporating gel, PLLA sponge, and cells would become a useful product.

On the other hand, attempts (5–10) at manufacturing artificial vascular grafts of collagen gel and cells had