Effect of Exogenous Extracellular Matrices on Proteoglycan Synthesis by Cultured Rabbit Costal Chondrocytes

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ABSTRACT We examined the effect of an extracellular matrix (ECM), produced by either bovine corneal endothelial (BCE) cells or mouse PF HR-9 teratocarcinoma cells, on the ability of rabbit costal chondrocytes to re-express their phenotype once confluent. Rabbit chondrocytes seeded at low densities and grown on plastic tissue culture dishes produced a heterogeneous cell population composed of both overtly differentiated and poorly differentiated chondrocytes, as well as fibroblastic cells. On the other hand, cultures grown on BCE-ECM- or HR-9-ECM-coated dishes reorganized into a homogeneous cartilage-like tissue composed of round cells surrounded by a refractile matrix that stained intensely with alcian green. The cell ultrastructure and that of their pericellular matrix were similar to those seen in vivo. The differentiation of chondrocyte cultures grown on the ECMs vs. plastic was reflected by a two- to three-fold increase in the maximal rate of incorporation of [^{35}S]sulfate and [^{3}H]glucosamine into proteoglycans. Furthermore, the ratio of ^{35}S-labeled proteoglycans incorporated in the cell layer vs. those released into the medium was 1.5–2.5-fold higher when cultures were grown on the ECMs than on plastic. This suggests that the ECMs stimulate the incorporation of newly synthesized proteoglycans into a cartilaginous matrix. Since chondrocyte cultures grown on BCE-ECM or HR-9-ECM give rise to a homogeneous cartilage-like tissue even when seeded at low cell densities, they provide a model for the study of cell-substrate interactions that are responsible for the maintenance of the differentiated phenotype of chondrocytes.

Chondrocyte cultures have been widely used as a model to study the control of the synthesis of extracellular matrix macromolecules (1–4). However, cartilage cells readily lose their phenotypic characteristics when grown on plastic tissue culture dishes (5–7), yielding at confluence, in most instances, a heterogeneous cell population composed of both differentiated chondrocytes and fibroblastic or epithelioid cells. This heterogeneous cell population has impaired the biological analysis of the mechanisms involved in the regulation of the synthesis of cartilage-specific macromolecules. The reasons for the heterogeneous cell differentiation observed in confluent chondrocyte cultures maintained on plastic culture dishes are not clear. It is known, however, that overtly differentiated chondrocytes are present only in cartilage nodules where cartilage matrix prevents contact between the cells and the plastic substratum. In contrast, fibroblastic cells are present only in the areas surrounding cartilage nodules and directly attach to the substrate (2, 5).

We examined the effects of the substrate on proteoglycan synthesis by cultured chondrocytes, using rabbit costal chondrocytes maintained on plastic vs. extracellular matrix (ECM)–coated dishes.[1] Our results indicated that rabbit cartilage cells seeded at low densities and grown on an ECM produced by bovine corneal endothelial (BCE) cells or mouse embryo PF-HR-9 teratocarcinoma cells give rise to a homogeneous cartilage-like tissue in confluent cultures. In contrast, similar cultures grown on plastic produced a heterogeneous cell population, including both overtly differentiated chondrocytes and fibroblastic cells. The differentiation of chondro-

[1] *Abbreviations used in this paper:* BCE cells, bovine corneal endothelial cells; DME, Dulbecco's modified Eagle's medium, FGF, fibroblast growth factor; ECM, extracellular matrix; HR-9 cells, mouse PF HR-9 teratocarcinoma cells; CPC, cetylpyridinium chloride; Δ Di-0S, 2-acetoamido-2-deoxy-3-*O*-(β-D-gluc-4-enepyranosyluronic acid)-D-galactose; Δ Di-4S, 2-acetoamido-2-deoxy-3-*O*-(β-D-gluc-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; Δ Di-6S, 2-acetoamido-2-deoxy-3-*O*-(β-D-gluc-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; Δ Di-HA, 2-acetoamido-2-deoxy-3-*O*-(β-D-gluc-4-enepyranosyluronic acid)-D-glucose; PBS, phosphate-buffered saline.

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cytes cultures maintained on ECM vs. plastic correlated with an increased rate of proteoglycan synthesis by cultures maintained on both types of the ECMs. In addition, the structures of proteoglycans produced by cultures maintained on the ECMs differed from those produced by cultures maintained on plastic.

MATERIALS AND METHODS

Materials: Fibroblast growth factor (FGF) was purified from bovine pituitary as previously described (8). Dulbecco's modified Eagle's medium (DME, H-16) was obtained from Gibco Laboratories, Grand Island, NY. Fetal calf serum was obtained from Hyclone Sterile System Inc., Logan, UT. Tissue culture dishes were from Falcon Labware, Oxnard, CA, gentamicin from Schering Corp., Kenilworth, NJ, and fungizone from E. R. Squibb & Sons, Princeton, NJ. [^{35}S]Sulfate (1,290 Ci/mmole), D-[6-^{3}H]glucosamine (21.1 Ci/mmol), and aquasol were purchased from New England Nuclear, Boston, MA; chondroitinase AC, chondro 4-sulfatase, chondro 6-sulfatase, and chondroitin sulfate were from Seikagaku Kogyo Co., Tokyo, Japan; pronase and papain were from Sigma Chemical Co., St. Louis, MO; Sepharose CL-2B and CL-6B were from Pharmacia Fine Chemicals, Piscataway, NJ.

Preparation of ECM-coated Dishes: Cultures of BCE cells were established from bovine eyes as previously described (9). BCE-ECM—coated dishes were prepared by treatment of confluent corneal endothelial cell cultures with 0.02 M NH_4OH in distilled water for 5 min, followed by washing with 0.01 M sodium phosphate (pH 7.4), 0.9% NaCl (phosphate-buffered saline [PBS]) as described previously (10, 11). HR-9 cells were seeded at 2×10^4 cells/35-mm dish on fibronectin-coated dishes and incubated for 4 d in 2 ml DME containing 10% fetal calf serum, 50 μg/ml gentamicin, and 0.25 μg/ml fungizone. The medium was replaced with 2 ml of the same medium, except that 5% fetal calf serum was substituted for 10% fetal calf serum. Cells were incubated for 6 additional d, and HR-9-ECM–coated dishes were then prepared by NH_4OH treatment as in the case of BCE-ECM–coated dishes (11). The HR-9 matrix is composed of collagen type IV, heparin sulfate proteoglycans, laminin, and entactin (12–14), and has a composition very close to that seen in vivo in capillary endothelial cell basement membrane (15, 16). In contrast, the BCE-ECM contains, in addition to the components present in the HR-9 matrix, elastin, collagen type I and III, fibronectin, and dermatan sulfate proteoglycans (17–19).

Chondrocyte Culture: Chondrocytes were isolated from rib cartilage of 3–4-wk-old male New Zealand rabbits as described by Shimomura et al. (20). Growth cartilage was dissected out and cut into small pieces (1–3 mm^3) with a scalpel. The pieces were incubated for 15 min at 37°C in 20 ml of 0.1% EDTA in PBS and then centrifuged at ~1,100 rpm in an International clinical centrifuge (Model CL, No. 221 head) for 5 min. The pellet was resuspended in 20 ml of 0.125% trypsin in PBS. After 30 min at 37°C, the tissue fragments were centrifuged as described above, and the pellet resuspended in 0.2% collagenase (Sigma Chemical Co., type IA) in PBS. After 2.5 h at 37°C, the tissue fragments and cell aggregates were aspirated up and down a 10-ml plastic pipette. The resulting cell suspension was filtered through a nylon sieve (pore size = 45 μm). The filtrate was centrifuged and the pellet was washed three times with DME supplemented with 50 μg/ml gentamicin and 0.25 μg/ml fungizone.

Cell Growth Measurement and Staining with Alcian Green of Cultured Chondrocytes: Chondrocytes were seeded at densities ranging from 1×10^2 cells to 1×10^5 cells/35-mm dish in 2 ml DME supplemented with 50 μg/ml ascorbic acid, 50 μg/ml gentamicin, 0.25 μg/ml fungizone, and 10% fetal calf serum. Cultures were maintained either on plastic tissue culture dishes or dishes coated with BCE-ECM or HR-9-ECM. After various periods of incubation, triplicate plates representing each culture condition were incubated for 2 h in 1 ml PBS containing 0.1% collagenase and 0.1% pronase. When cells were detached from the substratum, cell number was determined with a Coulter Counter (Coulter Electronics Inc., Hialeah, FL). The morphological appearance of the cultures was analyzed by phase-contrast microscopy and pictures were taken on day 18. In addition, the dishes were fixed and stained with alcian green, which stains the strongly anionic sulfated proteoglycans present in large amounts in cartilage matrix (21). After fixation in 10% formalin, the dishes were rinsed with water and treated with 3% acetic acid for 10 min. The cells were then stained for 10 min with a solution of 0.5% alcian green in 3% acetic acid. They were then rinsed with water, treated for 3 min with 3% acetic acid, and rinsed again in water for 3 min (22).

Ultrastructure of Chondrocyte Cultures: Chondrocyte cultures in 35-mm ECM-coated dishes were fixed for 1–2 h with 0.5% paraformaldehyde and 1% glutaraldehyde, buffered with a 0.1-M sodium cacodylate containing 0.2 mM $CaCl_2$ (pH 7.4). The cultures were postfixed with 1% osmium in 0.07 M veronal acetate for 1 h, dyhydrated in graded alcohol 50–100%, and embedded in Araldite 502. A JEOL 100C electron microscope was used to photograph thin sections of cell cultures that were stained with uranyl acetate and lead citrate (23).

Determination of the Rate of Proteoglycan Synthesis: After 6 to 24 d in culture, cells were exposed for 3 h at 37°C to 2 μCi/ml of [^{35}S]-sulfate in 0.8 ml DME. The rate of proteoglycan synthesis was determined by measuring incorporation of [^{35}S]sulfate into material precipitated with cetylpyridinium chloride, as previously described (24).

In another series of experiments, cultures were exposed for 3 h at 37°C to 175 μCi of [^{35}S]sulfate in 0.8 ml DME with 10% fetal calf serum. The medium was kept frozen at −30°C until analyzed. The cell layers were overlaid with 1.0 ml of buffer containing 4 M guanidine HCl, 0.1 M ε-amino-*n*-caproic acid, 20 mM EDTA, 1 mg/ml benzamidine HCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetic acid, and 50 mM Tris-HCl (pH 8.0). The culture dishes were then put on a shaker for 24 h at 4°C. After clarification by centrifugation, the cell extracts were stored at −30°C until analyzed.

Preparation of Rabbit Costal Cartilage Proteoglycan: Rabbit costal cartilage was prepared for extraction by mincing the cartilage with a scalpel. Proteoglycans were extracted from the tissue fragment by incubating them on a shaker platform for 24 h at 4°C with a solution of 4 M guanidine HCl in 50 mM Tris-HCl (pH 8.0) with protease inhibitors. Insoluble material was removed by centrifugation, and aliquots (2 ml) of the cartilage extracts were applied on a Sepharose CL-2B column (1.6 × 97 cm) equilibrated in 4 M guanidine HCl, 50 mM Tris-HCl buffer (pH 8.0) with protease inhibitors. 3-ml fractions were collected. Fractions 28–38, corresponding to the proteoglycan monomer, were pooled, and used as proteoglycan carrier.

Aggregation of Proteoglycans: Aliquots (0.16 ml) of the medium were mixed with aliquots (0.2 ml) of the appropriate cell layer fraction and 1.24 ml of water. After 1 h at 4°C, the solution was mixed with 0.1 ml of water containing 1 mg of rabbit costal cartilage proteoglycan. Proteoglycans were then precipitated by adding 3 vol (vol/vol) of 95% ethanol/1.3% potassium acetate. After 2 h at 0°C, the suspension was centrifuged, and the precipitate solubilized with 0.5 M sodium acetate (pH 7.0), 0.02 M sodium sulfate. The solution was applied onto a Sepharose CL-2B column (1.6 × 98 cm) equilibrated in 0.5 M sodium acetate (pH 7.0), 0.02 M sodium sulfate. Fractions were collected and counted as described in Fig. 6.

Relative Hydrodynamic Sizes of Proteoglycans: 0.4-ml aliquots of the medium were mixed with an equal volume of 8 M guanidine HCl in water. 0.3 ml of a 4-M guanidine HCl solution containing 1.5 mg rabbit costal cartilage proteoglycan was then added. Samples were applied onto a Sepharose CL-2B column (1.6 × 97 cm) equilibrated in 4 M guanidine HCl, 50 mM Tris-HCl buffer (pH 8.0) with protease inhibitors. Fractions were collected and counted as described in Fig. 7.

0.5-ml aliquots of the cell layer fractions were mixed with 0.3 ml of a 4-M guanidine HCl solution containing 1.5 mg rabbit costal cartilage proteoglycan carrier, and directly applied onto a Sepharose CL-2B column (1.6 × 97 cm) equilibrated in 4 M guanidine HCl, 50 mM Tris-HCl buffer (pH 8.0) with protease inhibitors. Fractions were collected and counted as described in Fig. 7.

Sepharose CL-6B Chromatography of Papain-digested Proteoglycan Monomer: Aliquots (4.5 ml) of the pooled fractions of the high molecular weight proteoglycan monomer eluting from Sepharose CL-2B were mixed with 9 ml of a water solution containing 2 mg chondroitin sulfate. 27 ml of 95% ethanol solution containing 1.3% potassium acetate was then added. After 2 h at 0°C, the suspension was centrifuged. The precipitate was washed with 70% ethanol, then dissolved with 0.15 ml of distilled water. Aliquots (100 μl) of the samples were mixed with 100 μl of water containing 100 μg of papain, 0.2 M sodium acetate, (pH 7.0), 10 mM EDTA, and 10 mM cysteine hydrochloride, and then incubated at 65°C for 5 h. The papain-digest was analyzed with a Sepharose CL-6B column (1.6 × 97 cm) that was equilibrated and eluted with 2 M guanidine HCl, 50 mM Tris-HCl (pH 8.0). Fractions were collected and counted as described in Fig. 8.

Labeling of Glycosaminoglycans with [^{3}H]Glucosamine and Preparation of Glycosaminoglycans: Chondrocytes were seeded (1×10^4 cells per 35-mm plastic or ECM-coated dish) in 2 ml DME supplemented with 50 μg/ml ascorbic acid, 50 μg/ml gentamicin, 0.25 μg/ml fungizone, and 10% fetal calf serum. After 14 d of incubation, cultures were exposed to 10 μCi/ml of [^{3}H]glucosamine in 1 ml DME supplemented with 50 μg/ml ascorbic acid, 50 μg/ml gentamicin, 0.25 μg/ml fungizone, and 10% fetal calf serum for 24 h. Medium was then removed, and the cell layers were scraped with a rubber policeman in 1 ml of ice-cold 0.1 M Tris-HCl, 2 mM $CaCl_2$ (pH 8.1). Both cell layers and medium were boiled for 5 min, and then mixed with 0.1 ml of 0.1 M Tris-HCl buffer (pH 8.1), 2 mM $CaCl_2$, and containing 1 mg pronase. The

solution was incubated for 3 d at 37°C. Fresh pronase (1 mg in 0.1 ml of 0.1 M Tris-HCl buffer [pH 8.1] containing 2 mM $CaCl_2$) was added daily. The reaction was stopped by boiling for 3 min, and insoluble material was removed by centrifugation. The supernatant was mixed with 1 mg chondroitin sulfate in 0.1 ml of water and then with threefold excess of 95% ethanol/1.3% potassium acetate. The samples were kept at 0°C for 1 h, and the resulting precipitate was collected by centrifugation. The precipitate was solubilized with 1.0 ml of water, and glycosaminoglycans were again precipitated by adding 3 ml of 95% ethanol/1.3% potassium acetate. This precipitation step was repeated five times to ensure complete elimination of low molecular weight materials. The final precipitate was solubilized with 0.3 ml of water.

Chondroitinase Digestion: The [^{3}H]glucosamine-labeled glycosaminoglycans prepared as described above were digested with chondroitinase (25). Aliquots (10–35 μl) of samples were mixed with 10 μl of water containing chondroitinase AC (0.2 U) and 5 μl of 1 M Tris-HCl buffer (pH 7.6) containing 0.05 M sodium acetate and 0.01 M NaF. In some experiments, aliquots of samples (25 μl) were mixed with 20 μl chondroitinase AC (0.2 U) and chondro 4- or 6-sulfatase (0.1 U), and 5 μl of 1 M Tris-HCl buffer (pH 7.6) containing 0.05 m sodium acetate. The reaction mixture containing 50 μl of the solution was incubated at 37°C for 5 h. After incubation, the mixture was heated at 100°C for 2 min. Aliquots (10–30 μl) of the solution were applied to a cellulose thin layer sheet (20 × 20 cm). A chromatography paper (Whatman No. 1) (Whatman Chemical Separation Inc., Clifton, NJ) extension was taped to the upper edge of the thin layer sheet to allow the solvent to rise 21–23 cm above the base line (26). After a 24-h desalting step using *n*-butanol:ethanol:water (52:32:16, vol/vol), chromatograms were developed with *n*-butanol:acetic acid:2 M NH_4OH (2:3:1) (26). Radioactivity in each fraction (0.5 cm) was eluted from the cellulose by addition of 0.5 ml of 0.1 M HCl followed by heating for 24 h at 65°C. Aquasol (5 ml) was added and radioactivity determined.

RESULTS

Effect of ECM on Proliferation of Chondrocytes

The growth of rabbit costal chondrocytes maintained on ECM produced by BCE cells or HR-9 cells was compared with that of similar cultures maintained on plastic. Freshly isolated chondrocytes from the ribs of young rabbits were seeded at a density of 10^4 cells/35-mm plastic culture dish coated or not with ECM and exposed to DME supplemented with 10% fetal calf serum. The plating efficiency was >90% in both types of culture. After a 2-d lag-time, cells in all cultures started to proliferate actively. Average doubling times during their logarithmic growth phase in cultures maintained on BCE-ECM and HR-9-ECM were 14 and 17 h, respectively, and were shorter than that (27 h) of cultures maintained on plastic (Fig. 1*A*). Cultures became confluent on days 6 and 7 for cells maintained on BCE-ECM and HR-9-ECM, respectively, and by day 10 for cells maintained on plastic. The final cell densities (1.5 × 10^6 cells/35-mm dish) of cultures maintained on the ECMs were 35% higher than that of cultures maintained on plastic (1.1 × 10^6 cells/35-mm dish). This improvement in the ability of chondrocytes to proliferate when maintained on BCE-ECM and HR-9-ECM was observed at seeding densities of 10^2 cells and 10^5 cells/35-mm dish (Fig. 1, *B* and *C*, respectively). The growth-promoting effect of BCE-ECM was slightly greater than that of HR-9-ECM, especially in low density cultures.

Addition of FGF purified from bovine pituitary glands (8) to chondrocyte cultures maintained on plastic or BCE-ECM–coated dishes resulted in marked increases in the growth rate and final cell density. The average population doubling time during the logarithmic growth phase of cultures maintained on plastic and exposed to FGF was 18 h as compared with 27 h in its absence. The average population doubling time of cultures maintained on BCE-ECM and exposed to FGF was 12 h as compared with 14 h in its absence (Fig. 1*A*). The final

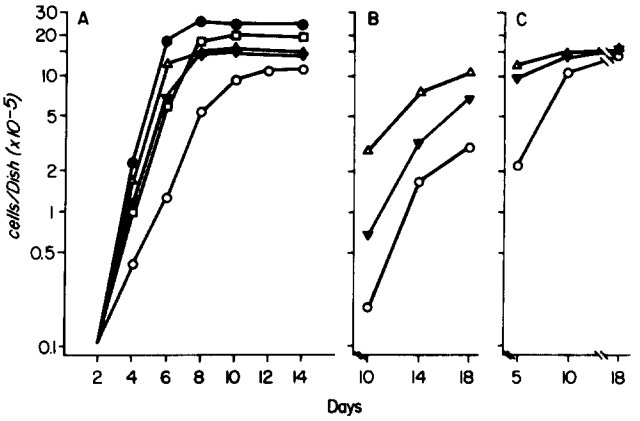

FIGURE 1 Comparison of the growth rate of low density chondrocyte cultures maintained on plastic culture dishes coated or not with BCE-ECM or HR-9-ECM and exposed to DME supplemented with 10% fetal calf serum in the presence or absence of FGF. Rabbit chondrocytes were seeded at 1 × 10^4 (*A*), 1 × 10^2 cells (*B*), or 1 × 10^5 cells (*C*) per 35-mm dish. Cells were maintained on plastic culture dishes in the absence (○) or presence (□) of FGF, on dishes coated with BCE-ECM in the absence (△) or presence (●) of FGF, or on dishes coated with HR-9-ECM in the absence of FGF (▼). FGF (1 ng/ml) was added every other day. On the days indicated, triplicate plates representing each condition were harvested and counted, as described under Materials and Methods. The standard deviation in the different determinations did not exceed 10% of the mean.

cell densities in cultures maintained on plastic and BCE-ECM and exposed to FGF were 2.0 × 10^6 cells and 2.5 × 10^6 cells/35-mm dish, respectively. These values were 1.7–1.8-fold higher than the final cell density of cultures maintained on plastic (1.1 × 10^6 cells/35-mm dish) or BCE-ECM (1.5 × 10^6 cells/35-mm dish) and not exposed to FGF.

Effect of BCE-ECM and HR-9-ECM on Morphological Appearence of Chondrocytes

When cartilage cells were seeded at a low density (10^4 cells/35-mm dish) on plastic tissue culture dishes, >50% of the cells in confluent cultures adopted a fibroblastic or epithelioid configuration. No conversion of the cells to overtly differentiated spherical chondrocytes was observed, even after 2–3 wk in culture (Fig. 2*A*). In contrast, almost all cells (>99%) in cultures grown on BCE-ECM (Fig. 2*B*) or HR-9-ECM (Fig. 2*C*) reassumed, at confluence, the spherical conformation characteristic of chondrocytes. These spherical cells were surrounded by a refractile matrix that stained intensely with alcian green (Fig. 2*D*). On the other hand, cultures grown on plastic stained poorly and only in the areas where cartilage nodules were observed (Fig. 2*D*). Although BCE-ECM and HR-9-ECM contained proteoglycan, they did not react with the stain (data not shown). This is in agreement with previous studies that demonstrated that alcian green staining is specific for strongly anionic sulfated proteoglycans present in cartilage matrix (21, 22). Transmission electron microscope analysis showed that chondrocyte cultures maintained on ECM-coated dishes had an ultrastructure characteristic of chondrocytes in vivo and were embedded in a three-dimensional cartilaginous matrix in which collagen fibrils were observed (Fig. 2*E*). The ultrastructure of the matrix was strikingly similar to that

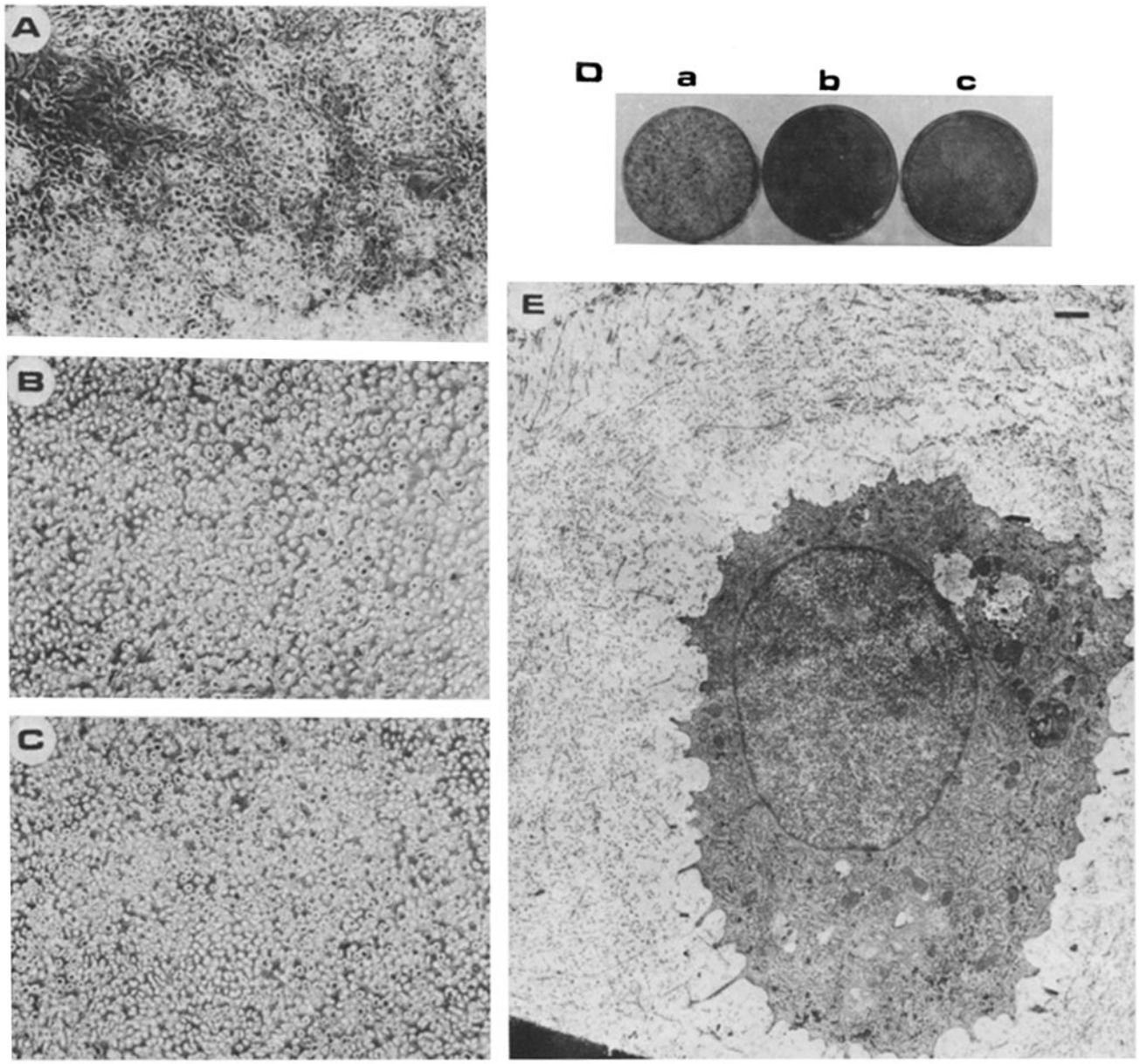

FIGURE 2 Morphological appearance of rabbit costal chondrocytes maintained on plastic culture dishes coated or not with BCE-ECM or HR-9-ECM and exposed to DME supplemented with 10% fetal calf serum. (*A–C*) Rabbit chondrocytes were seeded at 1 × 10^4 cells/35-mm dish on plastic culture dishes coated or not (*A*) with BCE-ECM (*B*) or HR-9-ECM (*C*) and exposed to DME supplemented with 10% fetal calf serum and antibiotics. Pictures were taken on day 18 with a Nikon phase-contrast photomicroscope (× 100). (*D*) Comparison of alcian green staining of rabbit costal chondrocyte cultures grown on plastic (*a*) vs. BCE-ECM- (*b*) or HR-9-ECM- (*c*) coated dishes. Culture conditions were as described above. After 18 d (*a–c*) in culture, one set of plates were fixed with 10% formalin and stained with alcian green, as described under Materials and Methods. (*E*) Transmission electron microscopy of chondrocyte culture maintained on BCE-ECM-coated dishes. A cross-section of the cell layer maintained for 16 d on BCE-ECM-coated dishes is shown. Culture conditions were as described in Fig. 2, *A–C*.

produced by chondrocytes in vivo (27).

Fig. 3 shows the changes in the morphology of chondrocytes during their proliferative and confluent stages when cultures are maintained on plastic dishes coated or not with BCE-ECM. When cultures were maintained on plastic, cells had a fibroblastic appearance during their growing stage (Fig. 3*A*, day 6), as well as during the early confluent stage (Fig. 3*B*, day 10). Although a small population of cells reassumed a spherical conformation on day 14 (Fig. 3*C*), >80% of the cells failed to re-express the spherical phenotype even after 19 d in culture. When cultures were maintained on BCE-ECM, cells adopted a fibroblastic or epithelioid conformation during the proliferative (data not shown) and early confluent stages (Fig. 3*D*, day 6). In contrast to cultures maintained on plastic, the cells maintained on ECM had few cellular processes. After 8 d in culture, they started to round up, forming cartilage nodules on day 10, composed of spherical cells (Fig. 3*E*). The number and size of cartilage nodules increased with the prolongation of the culture periods. Within 14 d in culture, cells reorganized into a homogeneous cartilage-like tissue (Fig. 3*F*). Similar morphological changes were observed in cultures maintained on HR-9-ECM. These observations suggest that,

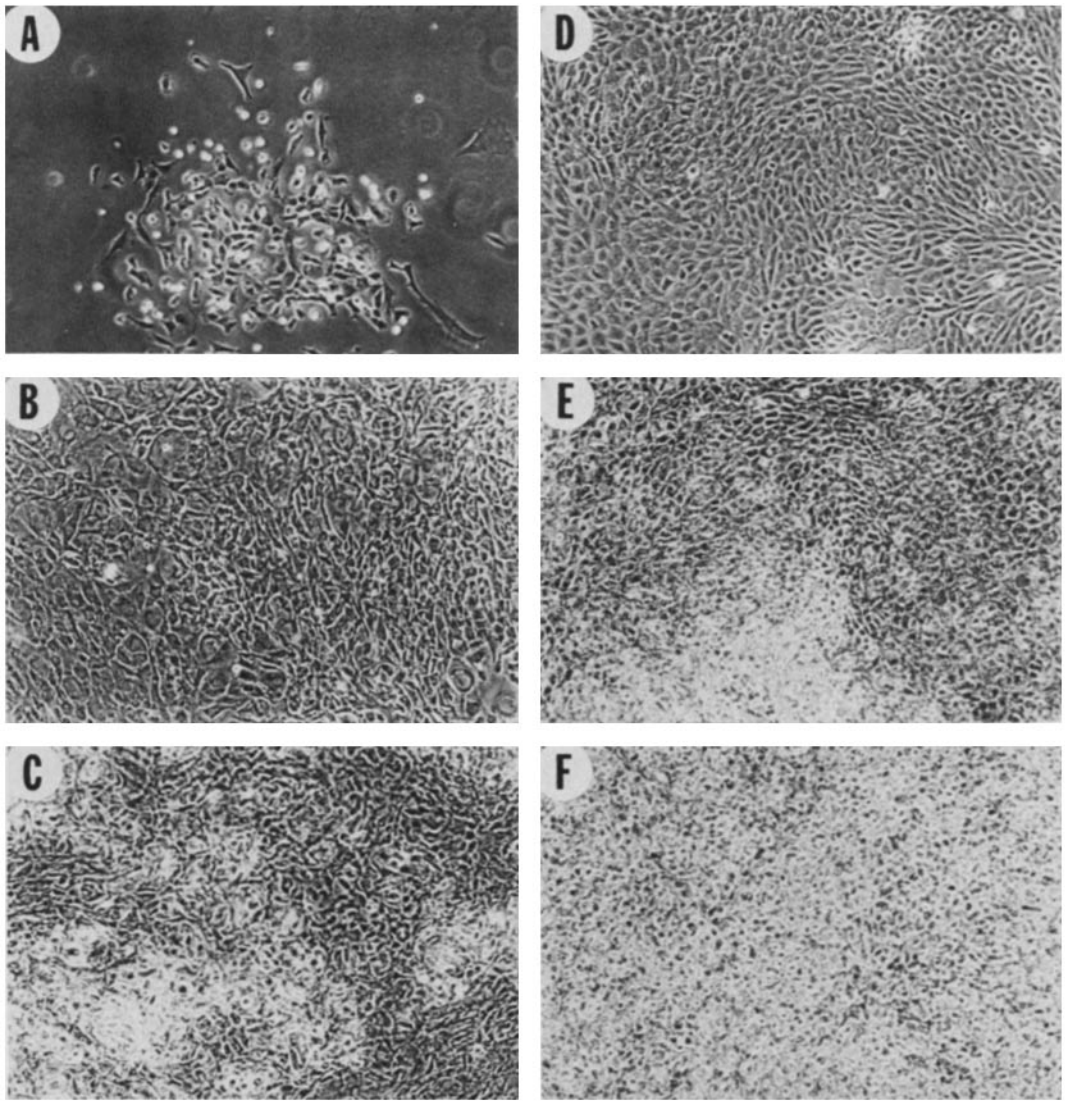

FIGURE 3 Morphological changes of chondrocytes maintained on plastic dishes coated or not with BCE-ECM. Chondrocytes were seeded and maintained on plastic dishes coated (*D–F*) or not (*A–C*) with BCE-ECM, as described in Fig. 2. Pictures were taken on day 6 (*A* and *D*), day 10 (*B* and *E*), and day 14 (*C* and *F*) with a Nikon phase-contrast photomicroscope (× 100).

even when maintained on ECM-coated dishes, chondrocytes temporarily lose their phenotypic expression during the proliferation and early confluent stages of culture, but, in contrast to cells maintained on plastic, will re-express it in the late confluent stage.

Effect of BCE-ECM and HR-9-ECM on Proteoglycan Synthesis by Chondrocytes

Fig. 4*A* shows the changes in the rate of [^{35}S]sulfate incorporation into proteoglycans present in the cell layers. The rate of [^{35}S]sulfate incorporation during the logarithmic growth phase was extremely low in all cultures. After 6 d in culture, the rate of [^{35}S]sulfate incorporation markedly increased, reaching a maximum on day 14 for cultures maintained on BCE-ECM or HR-9-ECM and on day 19 for cultures maintained on plastic. No decline in the maximum rate of [^{35}S]-sulfate incorporation was observed between day 14 or 19 depending on culture conditions (ECM or plastic) and day 24. The maximal level of incorporation of [^{35}S]sulfate in cultures maintained on BCE-ECM or HR-9-ECM was consistently two to threefold higher than that reached in cultures maintained on plastic in these and similar experiments with different batches of chondrocytes.

Fig. 4*B* shows the changes in the rate of [^{35}S]sulfate incorporation into proteoglycans that are released into the medium. The rate of incorporation of [^{35}S]sulfate into medium proteoglycans increased slowly after day 6, reaching a maximum on day 10 to 14 for cultures maintained on the ECMs and on day 14 for cultures maintained on plastic. Thereafter, it decreased in cultures maintained on the ECMs. In all cultures, [^{35}S]sulfate incorporation into medium proteoglycans was much less (<25%) than that into cell layer proteoglycans.

Fig. 4*C* shows the changes in the rate of [^{35}S]sulfate incorporation into proteoglycans per cell. On day 6, the rate of proteoglycan synthesis as reflected by [^{35}S]sulfate incorporation per cell in cultures maintained on BCE-ECM or HR-9-ECM was slightly lower than that in cultures maintained on plastic. After day 6, the rate of [^{35}S]sulfate incorporation per

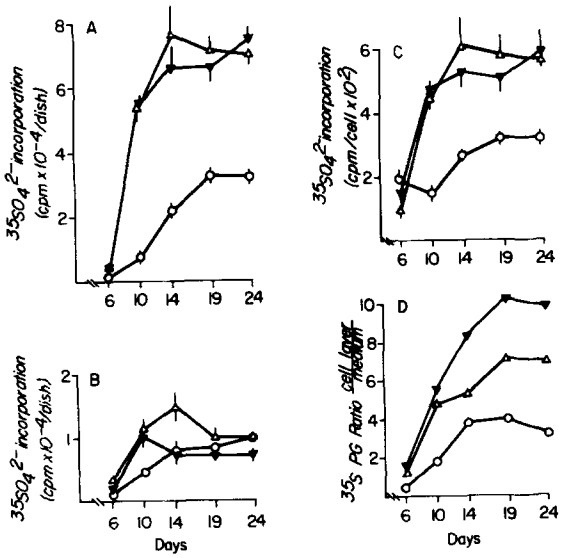

FIGURE 4 Changes in the rate of [^{35}S]sulfate incorporation into proteoglycans and distribution ratio between ^{35}S-labeled proteoglycans present in the cell layer vs. those released into the medium in low density chondrocyte cultures maintained on plastic, BCE-ECM, and HR-9-ECM, and exposed to DME supplemented with 10% fetal calf serum. Rabbit chondrocytes were seeded at 1×10^4 cells/35-mm dish on plastic culture dishes coated or not (○) with BCE-ECM (△) or HR-9-ECM (▼) and exposed to DME supplemented with 10% fetal calf serum and antibiotics. After 6–24 d in culture, triplicate plates representing each condition were exposed to 2 μCi/ml of [^{35}S]sulfate in 0.8 ml DME. The rate of [^{35}S]sulfate incorporation into proteoglycans present in the cell layer (*A*), the rate of [^{35}S]-sulfate incorporation into proteoglycans released into the medium (*B*), and the rate of [^{35}S]sulfate incorporation into total proteoglycans per cell (*C*) were determined. The distribution ratio between ^{35}S-labeled proteoglycans present in the cell layers vs. those released into the medium is shown in *D*. Points and bars represent averages ± standard deviation for triplicate determinations.

cell in cultures maintained on the ECMs increased, reaching a maximum on day 14. On the other hand, the rate in cultures maintained on plastic increased after day 10, reaching a maximum on day 19. The maximal level reached in the cultures maintained on BCE-ECM was almost the same as that reached in the cultures maintained on HR-9-ECM, but was higher by 1.8–2.5-fold than that in cultures maintained on plastic in repeated experiments with different batches of chondrocytes.

Next, the effect of BCE-ECM and HR-9-ECM on the distribution of newly synthesized proteoglycans incorporated into the cell layers vs. those released into the medium was examined. The ratio between [^{35}S]proteoglycans present in the cell layers and those present in the medium increased after day 6 in all cultures, reaching a maximum on day 19 (Fig. 4*D*). This increase was accompanied by the appearance of extensive refractile extracellular matrices, suggesting an increased deposition of proteoglycans in a cartilaginous matrix. The proportion of ^{35}S-labeled proteoglycans present in the cell layers of cultures maintained on the ECMs was 1.5–2.5-fold higher than that of cultures maintained on plastic (Fig. 4*D*). This observation suggests that both ECMs stimulate the incorporation of newly synthesized proteoglycans into a cartilaginous matrix. Although BCE-ECM and HR-9-ECM had a similar effect on incorporation of [^{35}S]sulfate into total proteoglycans (Fig. 4*C*), the ratio between ^{35}S-labeled proteoglycan present in the cell layers vs. those present in the medium was significantly greater during the whole length of the experiments in the case of cultures maintained on HR-9-ECM (Fig. 4*D*). This suggests a greater efficiency in incorporation of newly synthesized proteoglycans into the matrix in cultures maintained on HR-9-ECM.

Proteoglycan Synthesis in Cultures Seeded at Various Cell Densities

The phenotypic expression of cultured chondrocytes is known to be markedly influenced by the initial cell density (28). We therefore analyzed the degree to which BCE-ECM and HR-9-ECM could stimulate proteoglycan synthesis in chondrocyte cultures seeded at densities ranging from 10^2 to 10^5 cells/35-mm plastic, BCE-ECM–, or HR-9-ECM–coated dish when cells were exposed for 10–14 d to DME supplemented with 10% fetal calf serum. The final cell density in the cultures maintained on plastic was a direct function of their initial cell density (Fig. 1). The final cell densities of cultures grown on the ECMs were higher than those grown on plastic. This effect was best seen in cultures seeded at low densities (10^2–10^3 cells/35-mm dish) and marginal in cultures seeded at high densities (10^4–10^5 cells/35-mm dish) (Fig. 1). BCE-ECM and HR-9-ECM increased the incorporation of [^{35}S]sulfate into proteoglycans, but they did so preferentially, increasing the incorporation of newly synthesized [^{35}S]proteoglycans into the cell layers and associated matrix over that released into the medium. This effect was seen in both low and high density cultures (Fig. 5).

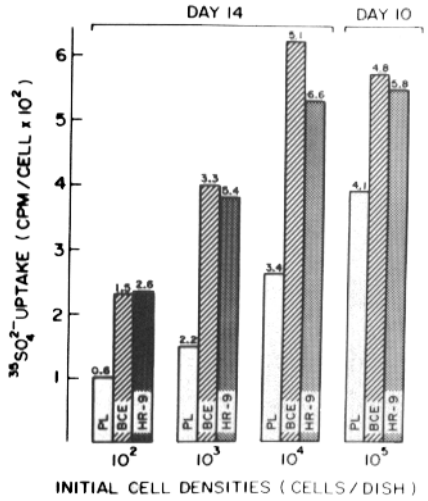

FIGURE 5 The rate of [^{35}S]sulfate incorporation into proteoglycans in rabbit costal chondrocyte cultures seeded at various densities and grown on plastic, BCE-ECM, or HR-9-ECM. Rabbit chondrocytes were seeded at densities ranging from 10^2 cells to 10^5 cells per 35-mm dish, and maintained on plastic culture dishes coated or not with BCE-ECM or HR-9-ECM. Cultures were exposed to DME supplemented with 10% fetal calf serum and antibiotics. After 10 to 14 d in culture, triplicate plates representing each condition were labeled with 2 μCi/ml of [^{35}S]sulfate for 3 h, as described under Materials and Methods. The distribution ratio between ^{35}S-labeled proteoglycans present in the cell layer vs. those released into the medium is shown on top of the bars. The standard deviation in the different determinations did not exceed 5% of the mean.

In another series of experiments using cultures seeded at densities ranging from 10^2 to 10^4 cells/35-mm dish, it was observed that BCE-ECM increases not only the incorporation of [^{35}S]sulfate into proteoglycans, but also that of [^{3}H]glucosamine into proteoglycans. Furthermore, even when seeded at a low initial density of 10^3 cells/35-mm dish, chondrocyte cultures maintained on BCE-ECM for >18 d developed a homogeneous cartilage-like tissue (data not shown).

Structure of Proteoglycan Synthesized by Chondrocytes Maintained on Plastic and ECM

To examine whether BCE-ECM and HR-9-ECM increase not only [^{35}S]sulfate incorporation but also that of [^{3}H]glucosamine into proteoglycans, we seeded rabbit costal chondrocytes at 10^4 cells/35-mm plastic, BCE-ECM–, or HR-9-ECM–coated dish and incubated them for 15 d in DME with 10% fetal calf serum. Cultures were exposed to 10 μCi/ml of [^{3}H]glucosamine in 1 ml DME supplemented with 10% fetal calf serum for the last 24 h of incubation. The level of radioactivity incorporated into glycosaminoglycans in cultures maintained on BCE-ECM (24.4 × 10^5 cpm/dish) or HR-9-ECM (22.0 × 10^5 cpm/dish) was threefold higher than that of cultures maintained on plastic (7.9 × 10^5 cpm/dish).

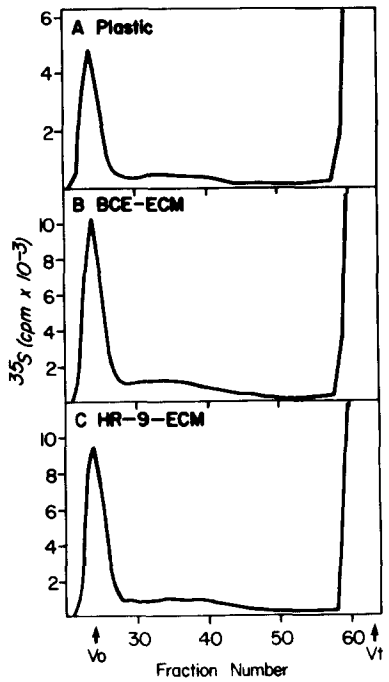

FIGURE 6 Sepharose CL-2B chromatography of proteoglycans from cultures grown on plastic, BCE-ECM, and HR-9-ECM under associative solvent conditions. Rabbit chondrocytes were seeded at 1 × 10^4 cells/35-mm dish on plastic culture dishes coated or not (*A*) with BCE-ECM (*B*) or HR-9-ECM (*C*) and incubated for 14 d. Proteoglycans were analyzed by gel exclusion chromatography on Sepharose CL-2B equilibrated with 0.5 M sodium acetate (pH 7.0), 0.02 M sodium sulfate. 3-ml fractions were collected. Aliquots (0.1 ml) of fractions were mixed with 0.1 ml of H_2O and 4 ml of Aquasol. The radioactivity was counted in a Beckman LS-8000 scintillation counter. *Vo* and *Vt* mark the column's void and total volumes, respectively. Free unincorporated radioactivity that was not completely removed by precipitation with ethanol/potassium acetate eluted at Vt.

This indicates that the two ECMs stimulate net synthesis of glycosaminoglycan chains. These results, however, do not eliminate the possibility that the increased incorporation of [^{35}S]sulfate and [^{3}H]glucosamine are due to the production of a proteoglycan that is not characteristic of cartilage matrix. To test this possibility, we analyzed the proteoglycan synthesized by chondrocytes maintained on plastic, BCE-ECM, and HR-9-ECM by gel filtration on Sepharose CL-2B.

Aliquots of the medium plus cell layer fractions precipitated with ethanol/potassium acetate were solubilized with 0.5 M sodium acetate, 0.02 M sodium sulfate (pH 7.0), and analyzed by gel exclusion chromatography on Sepharose CL-2B in associative solvent conditions with 0.5 M sodium acetate, 0.02 M sodium sulfate (pH 7.0) (Fig. 6). About 70% of the ^{35}S-labeled macromolecules synthesized by chondrocytes maintained on plastic, BCE-ECM, or HR-9-ECM eluted in the excluded volume, indicating that the majority of newly synthesized proteoglycans were able to form aggregates. The elution profiles of the labeled macromolecules from cultures maintained on BCE-ECM and HR-9-ECM were almost the same as that obtained from cultures on plastic. These results indicate that BCE-ECM and HR-9-ECM favor the synthesis of proteoglycan that forms aggregates.

The relative size of ^{35}S-labeled proteoglycans present in the cell layer vs. those released by chondrocytes into the tissue culture medium was analyzed as a function of substratum (either plastic, BCE-ECM, or HR-9-ECM) upon which cells were maintained. As shown in Fig. 6, *A–C*, the elution profile of ^{35}S-labeled proteoglycans present in the cell layer after exclusion gel chromatography on Sepharose CL-2B under dissociative solvent conditions, consists of a single broad peak characteristic of chondrocyte monomer proteoglycan. The ^{35}S-labeled proteoglycan monomers (K_{av} = 0.21–0.23) synthesized by chondrocytes grown on BCE-ECM or HR-9-ECM were similar, but were larger than those (K_{av} = 0.29) synthesized in chondrocytes grown on plastic (Fig. 7, *A–C*). The molecular size of the proteoglycans synthesized by the rabbit costal chondrocytes was comparable to that of cartilage-specific proteoglycans (K_{av} = 0.26) synthesized by rat chondrosarcoma cell cultures seeded at high density (1 × 10^6 cells/35-mm dish) and exposed to 15% fetal calf serum, when analyzed by Sepharose CL-2B chromatography (3).

Proteoglycans released into the culture medium were found to be of large hydrodynamic size and were similar to those isolated from the cell layers (Fig. 7, *D–F*). The proteoglycan monomers (K_{av} = 0.21–0.24) synthesized and released into the medium by chondrocytes maintained on BCE-ECM or HR-9-ECM were larger than those (K_{av} = 0.29) from cultures maintained on plastic. A small population of low molecular weight proteoglycans (K_{av} = 0.60) was also found in the medium fractions from cultures maintained on plastic and ECMs.

When aliquots of the cell layer fractions and the medium from cultures maintained on plastic and ECMs were analyzed by gel exclusion chromatography on Sepharose CL-6B equilibrated with 2 M guanidine HCl, 50 mM Tris-HCl (pH 8.0), no free ^{35}S-labeled glycosaminoglycan chains were found in any cultures (data not shown). Therefore, the increase in [^{35}S]-sulfate incorporation in cultures maintained on BCE-ECM or HR-9-ECM was not due to synthesis of free glycosaminoglycan chains.

The increase in the hydrodynamic size of the large proteoglycan (K_{av} = 0.19–0.24) in cultures maintained on BCE-

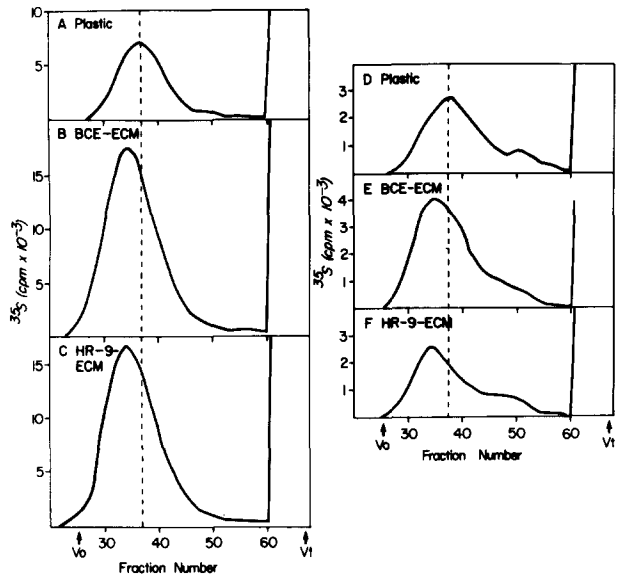

FIGURE 7 Sepharose CL-2B chromatography of proteoglycans located in the cell layers and in the medium from cultures grown on plastic, BCE-ECM, and HR-9-ECM under dissociative solvent conditions. Cultures were seeded and maintained for 14 d as described in Fig. 6. Aliquots of the 4-M guanidine HCl extract of the cell layers (*A–C*) or aliquots of the medium (*D–F*) from cultures grown on plastic (*A* and *D*), BCE-ECM (*B* and *E*), or HR-9-ECM (*C* and *F*) were applied on a column of Sepharose CL-2B that was equilibrated in 4 M guanidine HCl, 50 mM Tris-HCl (pH 8.0) with protease inhibitors as described under Materials and Methods. 3-ml fractions were collected. Aliquots (0.1 ml) of each fraction were mixed with 0.1 ml ethanol and 4 ml Aquasol. Radioactivity was measured in a Beckman LS-8000 scintillation counter. Free unincorporated radioactivity elutes at *Vt*. *Vo* was determined with high molecular weight hyaluronic acid synthesized by rabbit costal chondrocytes.

ECM or HR-9-ECM relative to that present in cultures maintained on plastic (K_{av} = 0.26–0.31) was consistently observed on both day 14 and day 19 with different batches of chondrocytes. Furthermore, this effect of the ECMs was seen in both low (10^3 cells/35-mm dish) and high (10^5 cells/35-mm dish) density cultures (data not shown).

Molecular Weight of Newly Synthesized Glycosaminoglycans

The ^{35}S-labeled glycosaminoglycans, cleaved from the intact large proteoglycans by treatment with papain, eluted in the included column volume on Sepharose CL-6B (Fig. 8). The size of the glycosaminoglycan chains associated with high molecular weight proteoglycan synthesized by chondrocytes maintained on BCE-ECM and HR-9-ECM (K_{av} = 0.51–0.53) was slightly larger than that observed when cells were maintained on plastic dishes (K_{av} = 0.56). It is unlikely, however, that the small increase in the glycosaminoglycan chain size can by itself account for the 1.8- to 2.5-fold increase in [^{35}S]-sulfate incorporation in cultures maintained on the ECMs.

Chondroitinase Digestion

The [^{3}H]glucosamine-labeled glycosaminoglycans from cultures maintained on plastic and ECM were digested with chondroitinase AC in the presence or absence of chondro 4-sulfatase or chondro 6-sulfatase, and the released disaccharides were analyzed by thin layer chromatography, as described under Materials and Methods. Treatments of [^{3}H]-glucosamine-labeled glycosaminoglycans with chondro 6-sulfatase and chondro 4-sulfatase in the presence of chondroitinase AC almost completely released radioactivity from the fractions of ΔDi-6S[1] and ΔDi-4S, respectively, and produced an equivalent amount of ΔDi-0S (data not shown). Therefore, the fractions indicated really represents ΔDi-6S, ΔDi-4S, and ΔDi-0S (Table I). Chondroitinase AC depolymerizes both chondroitin sulfate and hyaluronic acid (29). The unsaturated disaccharide (ΔDi-HA) released from hyaluronic acid was also analyzed by thin layer chromatography by the method of Mason et al. (26). In samples from cultures maintained on plastic- and ECM-coated dishes, >93% of the total [^{3}H]-glucosamine-labeled glycosaminoglycans were digested to disaccharide by chondroitinase AC (Table I). These findings indicate that maintaining chondrocytes on the ECMs does not result in production of proteoglycans containing other types of glycosaminoglycans, such as heparan sulfate and dermatan sulfate.

In samples from cultures maintained on BCE-ECM or HR-9-ECM, >70% of the radioactivity migrated with the ΔDi-6S standard, indicating chondroitin sulfate chains were primarily 6-sulfated. On the other hand, only 43–56% of the radioactivity in samples from cultures maintained on plastic migrated with the ΔDi-6S standard (Table I). The 6S/4S disaccharide ratio of ^{3}H-labeled glycosaminoglycans from the cultures maintained on the ECMs was 4.9–5.8. On the other hand, the 6S/4S ratio for samples from cultures maintained on plastic was 3.3–4.7 (Table I). This observation indicates that both ECMs influence the position of sulfation of chondroitin sulfate. Furthermore, 5.0–6.4% of the radioactivity in samples from cultures maintained on BCE-ECM or HR-9-ECM migrated with the ΔDi-0S standard, as compared with 10.7–10.8% for samples from cultures maintained on plastic (Table I). This suggests that both ECMs alter the sulfation of the chondroitin sulfate glycosaminoglycans to a small degree. Although cultured rabbit articular chondrocytes synthesize oversulfated chondroitin sulfate under certain experimental conditions (30), disulfated disaccharides were not observed at significant levels in samples from rabbit costal chondrocyte

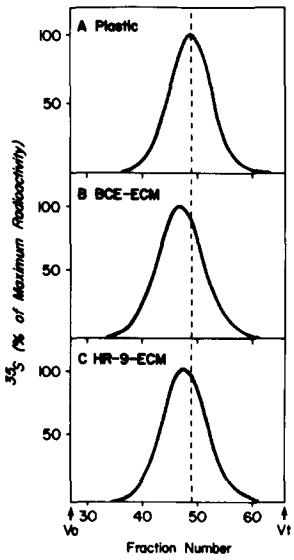

FIGURE 8 Sepharose CL-6B chromatography of papain-digested proteoglycan monomer. Glycosaminoglycans were liberated from high molecular weight proteoglycans located in the cell layers of cultures grown for 15 d on plastic (*A*), BCE-ECM (*B*), or HR-9-ECM (*C*) by treatment with papain, as described under Materials and Methods. Cultures were seeded and maintained as described in Figs. 5 and 6. Glycosaminoglycans prepared from the cultures were applied on a column of Sepharose CL-6B which was equilibrated in 2 M guanidine HCl, 50 mM Tris-HCl (pH 8.0). 2.5 ml of fractions were collected. Aliquots (0.5 ml) of fractions were mixed with 0.5 ml ethanol and 10 ml Aquasol. The radioactivity was counted in a Beckman LS-8000 scintillation counter. *Vo* and *Vt* were determined with blue dextran and [^{35}S]sulfate, respectively.

TABLE I

Disaccharide Analysis of Chondroitinase AC-digested Glycosaminoglycans

Substrate	Fraction	[^{3}H]Glucosamine uptake					
			Percent (%) distribution				
		cpm/cell × 10^3	Origin	ΔDi-6S	ΔDi-4S	ΔDi-OS	ΔDi-HA
Plastic	Cell layer	457	5.6	56.0	11.8	10.7	10.8
Plastic	Medium	198	7.0	43.2	13.1	10.8	23.4
BCE-ECM	Cell layer	969	2.4	71.2	12.3	5.5	4.1
BCE-ECM	Medium	496	2.3	71.5	12.5	6.0	3.7
HR-9-ECM	Cell layer	1,026	2.7	70.9	14.5	5.0	3.9
HR-9-ECM	Medium	295	2.3	69.9	12.0	6.4	4.6

[^{3}H]Glucosamine-labeled glycosaminoglycans from cultures maintained on plastic, BCE-ECM, or HR-9-ECM on day 15 were digested with chondroitinase AC, as described under Materials and Methods. Culture conditions were as described in Fig. 6.

cultures used in the present study. Therefore, the increased incorporation of [^{35}S]radioactivity in cultures maintained on the ECMs was not the result of synthesis of oversulfated chondroitin sulfate. Table I also shows that in samples from cultures maintained on BCE-ECM or HR-9-ECM, 3.7–4.6% of the radioactivity migrated with the ΔDi-HA standard, as compared with 10.8 to 23.4% in samples from cultures maintained on plastic.

DISCUSSION

Chondrocyte cultures have been used extensively during the past thirty years to study the control of their phenotypic expression (1). Such cultures are ordinarily maintained on plastic or glass tissue culture dishes. It has repeatedly been reported that chondrocytes grown on these artificial substrates progressively transform into motile cells that are morphologically indistinguishable from fibroblasts (5, 7, 31). It has been suggested that the mechanisms involved in chondrocyte dedifferentiation could be related to (*a*) overgrowth by contaminant cell types, (*b*) acquisition of new differentiation properties and loss of their proper ones in response to the in vitro conditions, and (*c*) chondrocyte aging in vivo (5, 6, 31, 32). However, the reasons for chondrocyte dedifferentiation in monolayer cultures have not been fully understood.

In the present study, we have shown that rabbit costal chondrocytes seeded at low cell densities (10^2–10^3 cells/35-mm dish) on plastic tissue culture dishes and exposed to DME supplemented with 10% fetal calf serum lose their phenotypic expression, as shown by their fibroblastic morphology and decreased incorporation of [^{35}S]sulfate after proliferating for 9 to 13 generations. In contrast, the majority of chondrocytes in cultures maintained on ECM-coated dishes retain their ability to express cartilage phenotype even after proliferating for more than 12 generations. This was reflected in confluent cultures by the ability of chondrocytes to reorganize a cartilage-like tissue in vitro, as shown by increases in the number of spherical cells, the extent of staining with alcian green, the incorporation of [^{35}S]sulfate and [^{3}H]glucosamine into proteoglycans, and the distribution of newly synthesized proteoglycans in the cell layer vs. those in the medium. Furthermore, the ultrastructure of a cartilage-like tissue in cultures maintained on ECM-coated dishes was similar, when analyzed by transmission electron microscopy, to that of the cartilage in vivo. These observations suggest that the dedifferentiation of chondrocytes grown on plastic tissue culture dishes reflects an adaptation to the artificial substrate rather than a mimicking of the in vivo aging process.

The present study also demonstrates that, regardless of substrate, cultured rabbit costal chondrocytes synthesize cartilage-characteristic proteoglycans that are able to form aggregates. Although the chondrocytes synthesized two species of proteoglycans (high and low molecular weight), the ratio of the large proteoglycan to the small one is above 10:1. The large proteoglycan has been shown to represent unique expression of the chondrogenic phenotype and the small proteoglycan is found in both chondrogenic and nonchondrogenic cells (3, 33, 34). BCE-ECM and HR-9-ECM markedly increased the incorporation of [^{35}S]sulfate into the large proteoglycan, but had little effect on its incorporation into the small proteoglycan. Furthermore, the ECMs increased the large proteoglycan monomer size and chondroitin sulfate chain size. These results suggest that ECMs act on cultured chondrocytes to increase the synthesis of a cartilage-specific proteoglycan that is larger than that produced by chondrocytes maintained on plastic. Stevens and Hascall (3) also showed that insulin and somatomedin increase the proteoglycan monomer size and chondroitin sulfate chain size when the hormones enhance net synthesis of cartilage-specific proteoglycan in rat chondrosarcoma cell cultures. These observations suggest that both hormones and extracellular matrix macromolecules influence the synthesis of cartilage-specific proteoglycan by chondrocytes.

The mechanism by which the ECMs promote or stabilize the expression of cartilage phenotype by cultured chondrocytes is not clear. Certain macromolecules, such as glycosaminoglycans and collagen, present in both the ECMs and cartilage matrix may be involved in the stimulation or stabilization of chondrocytes' phenotypic expression by the ECMs. Although these macromolecules present in the BCE-ECM and HR-9-ECM differ in their biochemical properties from those of cartilage matrix, the requirements for stimulation or stabilization of chondrocytes' phenotypic expression may not be highly specific. In any case, rabbit costal chondrocytes maintained on BCE-ECM and HR-9-ECM seem to provide a good experimental system for studies on the control of the phenotypic expression by chondrocytes, since they give rise to a homogeneous cartilage-like tissue even when seeded at low cell densities.

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