Culture of chondrocytes in alginate and collagen carrier gels

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In this in vitro study, we compared the potential of collagen and alginate gels as carriers for chondrocyte transplantation and we studied the influence of demineralized bone matrix (DBM) on chondrocytes in the gels. Chondrocytes were assessed for cell viability, phenotype (histology), proliferation rate and sulfate incorporation.

Collagen gels showed a significant increase in cell numbers, but the chondrocytes dedifferentiated into fibroblast-like cells from day 6 onwards. In alginate gels, initial cell loss was found, but the cells maintained their typical chondrocyte phenotype. Although the total quantity of proteoglycans initially synthesized per cell in collagen gel was significantly higher, expressed per cell, the quantity in alginate gel eventually surpassed collagen. No effects of culturing chondrocytes in combination with DBM could be demonstrated on cell proliferation and sulfate incorporation.

The collagen and alginate gels have different advantages as carriers for chondrocyte transplantation. The high proliferation rate of chondrocytes in collagen gel may be an advantage, but the preservation of the chondrocyte phenotype and the gradually increasing proteoglycan synthesis in alginate gel is a promising method for creating a hyaline cartilage implant in vitro.

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The classical procedure for repairing defects in hyaline articular cartilage is Pridie drilling; the subchondral bone is perforated to allow undifferentiated cells from the medullary cavity to enter the defect. However, the fibrous or fibrocartilaginous tissues formed are often of poor mechanical quality (Czitrom et al. 1986, Mankin 1986, Kim et al. 1991, Katoh and Urist 1993).

Transplantation of isolated chondrocytes into cartilage defects, on the other hand, seems a promising method for regenerating hyaline cartilage (Chesterman and Smith 1968, Bentley and Greer 1971, Green 1977, Aston and Bentley 1986, Grande et al. 1989, Wakitani et al. 1989, 1994). To optimize these grafting procedures, chondrocytes can be encapsulated in a delivery substance. The suitability of collagen (Speer et al. 1979, Yasui et al. 1982, Kimura et al. 1984, Wakitani et al. 1989, 1994, Schuman et al. 1995), fibrin glue (Itay et al. 1987, Homminga et al. 1993), hyaluronic acid (Robinson et al. 1990) and alginate (Guo et al. 1989, Atalata et al. 1993, Häuselmann 1994) have been evaluated in vitro. Irrespective of the type of implant, there is no satisfactory method as yet for fixing a transplant to subchondral bone.

Demineralized bone matrix (DBM) has demonstrated substantial osteoinductive activity, particularly in small laboratory animals (Urist et al. 1975, 1979, Glowacki et al. 1981a, b, Reddi 1981, Sakano et al. 1993). This capacity is probably based on growth factors (e.g., bone morphogenetic proteins; BMPs) which are present in DBM (Iwata et al. 1993, Jortikka et al. 1993, Katoh and Urist 1993, Nathanson 1994). The osteo- and chondrogenic potential of DBM in a synovial environment has been confirmed in animal experiments by placing DBM in cartilage defects (Billings et al. 1991, Dahlberg and Kreicbergs 1991). Although the defects were not completely filled with repair tissue and not all the tissues were hyaline cartilage, a good fixation of DBM to the subchondral bone was observed. Thus, theoretically, culturing chondrocytes on top of DBM has two potential advantages; the growth factors deriving from the DBM may stimulate chondrocyte proliferation and maintenance of the typical chondrocyte phenotype and, secondly, DBM may enhance the consolidation of the implant to the subchondral trabecular bone.

In this in vitro study, we focused on the potential of collagen type I and alginate gels as carriers of chondrocytes in future transplantation studies and we tested the effects of DBM on chondrocyte behavior in the two culture systems. Alginate, a linear polysaccha-

ride isolated from brown algae, is a linear co-polymer of two uronic acids, L-guluronic and D-manuronic acid linked by ß1,4 and α1,4 glucoside bonds. In the presence of divalent cations such as Ca^{++}, the polymer undergoes instant ionotrophic gelation (Guo et al. 1989).

Material and methods

Preparation of DBM

DBM was prepared from rabbit long bones, according to the method described by Urist (Urist et al. 1975, 1979). Rabbits were used because the quantity of DBM for each culture could easily be standardized. Both tibias and femurs from each animal were dissected under sterile conditions and, after removal of the epiphyses, periosteum and bone marrow, the specimens were cut into pieces of 1.5 cm. The bones were demineralized with 0.6N HCl for 24h at 4 °C and continuous stirring. Samples were rinsed repeatedly with deionized water at 4 °C until the pH of the wash matched that of water. They were then defatted with a 1:1 mixture of chloroform and methanol at 4 °C for 1 h and then rinsed again. Finally, the preparations were lyophilized and stored on silica gel under sterile conditions.

Chondrocyte isolation

Cartilage was harvested under sterile conditions from bovine metacarpophalangeal joints. Two joints from different animals were used for each experiment in order to reduce inter-experimental variation. Cells were isolated by incubation for an initial 2 h period in RPMI DM culture medium (Flow Laboratories, Irvine, UK; supplemented with 1 mM pyruvate and 1.2% gentamycin) with 0.2% pronase E (Sigma, St. Louis, MO, U.S.A.), followed by overnight incubation in a medium with 0.1% collagenase B (0.94 I.U./mg, Boehringer Mannheim, Germany), at 37 °C, with 95% air and 5% CO_2. After incubation, the slices of cartilage had almost completely been digested. Undigested fragments were removed by passing the solution through a nylon mesh. Isolated cells were washed 3 times by centrifugation and resuspended in 30 mL RPMI DM. Cell number and viability were assessed in a hemocytometer after staining with trypan blue. To obtain the appropriate cell number for culturing, a calculated volume of solution was separated and centrifuged.

Cultures in collagen gel

Collagen type I was isolated from rat tail tendon (Schor 1980, Schuman et al. 1995). Cells were dispersed in 7 volumes of cooled collagen solution (8 mg/mL in 0.05% acetic acid, ultraviolet light sterilized), two volumes of 3× concentrated DMEM medium and one volume of 0.2 M Hepes (Boehringer Mannheim, Germany) to a concentration of 2×10^6 cells/mL. 24 Wells plates were precoated with 0.3 mL collagen gel. 3 pieces of DBM (4 mm diameter and about 0.5 mm thick) were punched out and placed on the bottom half of the culture discs: 0.5 mL samples containing chondrocytes were put on top and allowed to gel at 37 °C. Gels were overlaid with 2.0 mL RPMI DM containing 10% fetal calf serum (FCS). The medium was changed every other day.

Cultures in alginate gel

Low viscosity alginate (1.2%; Keltone LV, Kelco, Chicago, IL, U.S.A.) was dissolved in physiological saline and autoclaved at 115 °C for 20 min. Pelleted chondrocytes were suspended in the alginate solution (2×10^6 cells/mL) and sampled as described for collagen. The alginate-chondrocyte suspension was allowed to gel by carefully adding a small aliquot of 102 mM $CaCl_2$. Gelation occurred instantly and after removal of the remaining $CaCl_2$, the gel was washed 4 times with physiological saline. DME/Ham's F12 (1:1) culture medium (Gibco, Grand Island, NY, U.S.A.) was used, supplemented with 10% FCS.

Sample analyses

Cell morphology was examined daily, using an inverted microscope. On days 2, 6, 9, and 12, cells were harvested to assess growth rate, phenotypic expression, viability and matrix production. At each time interval, 3 samples from different wells were obtained to estimate each metabolic parameter. Examinations were performed 4 times with chondrocytes from 2 different animals in each experiment to ensure reproducibility.

Growth rate and viability

Chondrocytes were recovered from the collagen gels by incubation with 1.0 mL 0.1% collagenase B in RPMI DM for 120 min at 37 °C. Alginate was dissolved by the addition of 1.0 mL 55 mM sodium citrate in 0.15 M NaCl. The recovered cells were counted in a hemocytometer and their viability was estimated with the trypan blue exclusion test.

Matrix production

To quantify proteoglycan synthesis as a measure of matrix production, cultures were overlaid with fresh medium containing [^{35}S]sulfate (Du Pont de Nemours, 's-Hertogenbosch, the Netherlands) at a final concentration of 5 µCi/mL. After an incubation

period of 3h, gels were separated from the medium and stored at –70 °C until required. After thawing, gels were dissolved, using either collagenase B or sodium citrate, as described previously. Cetylpyridinium chloride (CPC, Sigma; 0.1%) was added to the disintegrated gels and incubated overnight at 37 °C. The precipitate was washed with 0.05% CPC until the supernatant showed less than 100 cpm of radioactivity. The pellets of precipitated and washed proteoglycans were solubilized with Lumasolve (Perstorp Analytical, Oud-Beijerland, The Netherlands) for 3 h at 60 °C and analyzed, using liquid scintillation counting. From the total amount of proteoglycans synthesized, as determined with ^{35}S-incorporation in combination with the cell number per gel, we could also calculate the matrix production per cell.

Histology

For light microscopy, gels were fixed in a 0.1 M phosphate-buffered (pH 7.4) solution of 1% paraformaldehyde and 1.25% glutaraldehyde, dehydrated in alcohol and embedded in polymethylmethacrylate. Sections (7 µm) were stained with hematoxylin eosin (HE) and alcian blue. For electron microscopy, pellets were fixed in a mixture of 0.2% alcian blue, 0.2% ruthenium red, 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). After 4 hrs, the gels were rinsed in the same buffer and postfixed in the same mixture, but without glutaraldehyde. After a second rinse in the buffer, the gels were postfixed in 1% OsO_4, 0.2% alcian blue, 0.2% ruthenium red and 0.05 M cacodylate buffer for 4 hrs. Subsequently the gels were rinsed again in buffer, dehydrated with alcohol and embedded in Epon 812. To prevent the alginate beads from dissolving, 5 mM $CaCl_2$ was added to all solutions. Semi-thin sections (2 µm) were counterstained with toluidine blue. Adjacent ultra-thin sections were mounted on oval 2 mm one-hole grids, stained with uranyl acetate and lead citrate and examined with a Philips 301 electron microscope.

Statistical analysis of the results was performed using the rank sum t-test.

Results

Cell number and viability

The viability of chondrocytes in collagen gel was in excess of 95% directly after isolation. The gels showed an increase ($p < 0.01$) in the number of cells with time (Figure 1). In alginate gels, the viability was initially 80–90% on day 2, but the number stayed stationary thereafter (Figure 1). Culturing on top of

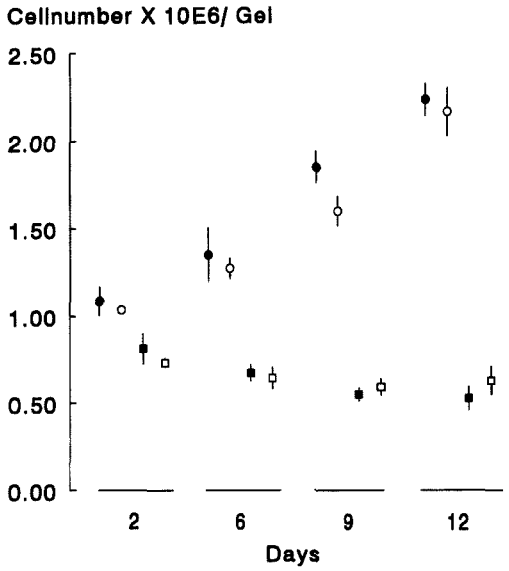

Figure 1. Growth curve of chondrocytes in collagen with (●) and without (○) DBM or alginate with (■) and without (□) DBM, plotted against culturing time. A typical example of 1 out of 4 experiments is presented. At least 3 samples were obtained after each time interval. Note the increase in cell number in collagen gel.

DBM had no effects on the growth rate or viability of the chondrocytes (Figure 1).

Incorporation study

Larger quantities ($p < 0.01$) of proteoglycans were synthesized from chondrocytes cultured in collagen than in alginate gels (Figure 3). In collagen gels ^{35}S incorporation increased with time for 12 days and gradually reached a stationary phase. A similar trend was found in alginate, but it started at a lower value. The addition of DBM caused no increase in matrix production in both types of gel (Figure 2).

Initially, more proteoglycans, expressed per chondrocyte, were synthesized from chondrocytes in collagen gel than in alginate gel (Figure 3). However, in collagen gel, matrix production decreased from day 6, whereas in alginate gel on day 6 it was still increasing. Subsequently, proteoglycan synthesis in alginate gel, expressed per chondrocyte, surpassed that in collagen gel on day 6. Again DBM showed no improvement in culturing conditions.

Morphology

Daily assessment with an inverted microscope and histology showed that the isolated chondrocytes were homogeneously distributed throughout the gels after suspension. Initially, chondrocytes had the same typ-

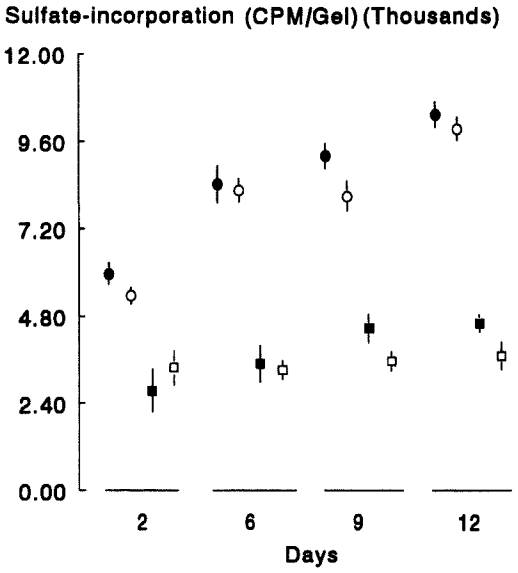

Figure 2. Typical example of the quantity of proteoglycans synthesized against time determined with ^{35}S incorporation, for collagen with (●) and without (○) DBM and alginate with (■) and without (□) DBM. At least 3 samples were examined at each time interval.

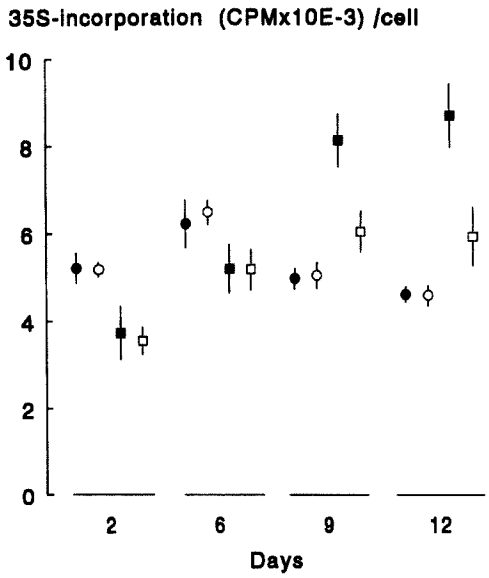

Figure 3. Proteoglycan synthesis per cell plotted against culture time for collagen with (●) and without (○) DBM and alginate with (■) and without (□) DBM. Results calculated from the data obtained from Figures 1 and 2.

ical polygonal shape in both gels. After 2 days of culture, many of the cells had become surrounded by a small territorial matrix which stained with alcian blue (Figure 4). The size of this region increased slightly until day 6. However, from day 6 onwards, morphological changes became apparent in the collagen gel. Chondrocytes gradually flattened, formed elongated cytoplasmatic extensions and dedifferentiated into fibroblast-like cells, with little or no matrix around them. Clusters of chondrocytes were scarce in the collagen gels.

In alginate, chondrocytes maintained their characteristically differentiated, rounded, chondrocyte morphology throughout the culture period. Initially, a fairly large percentage of the cells in culture was shrunken with necrotic cytoplasm and pycnotic nuclei. No matrix staining was observed around these cells. The alginate directly surrounding the viable cells after 2 days showed a diffuse blue staining. Alcian blue staining around the cells increased both in surface area and in intensity throughout the culture time. After 6 days, many cell clusters appeared. Directly around the cells in the clusters, the produced matrix showed a more irregular pattern, as if the produced components were aggregated (Figure 4).

Electron microscopy confirmed these observations. During the culture period, an irregular, nonstructured matrix had formed around the chondrocyte cultures in collagen gel. From day 6 onwards, the cells showed long cytoplasmic extensions and were surrounded by smaller quantities of matrix. In alginate gels, the cells maintained their rounded appearance. As the duration of the culture time increased, the intensity of the alginate gel staining increased due to matrix production. Occasionally, collagen-like structures were found in the matrix (Figure 5).

Discussion

Although collagen type II, instead of type I, is manly present in hyaline cartilage, type II collagen gels do not give better results as a delivery substance (Malemud et al. 1994). We are not sure whether dedifferentiation will have a negative influence on implant behavior after transplantation. However, it is known that a loss of chondrocyte morphology results in compromised matrix production (inferior quantity and biomechanical quality; Aulthouse et al. 1989). On the other hand, the chondrocyte proliferation rate in collagen gel is high and as it has been found that dedifferentiated cells can retain their original phenotype if placed in the correct environment (Coon 1966, Buschmann et al. 1992, Wakitani et al. 1994), dedifferentiation with a high proliferation rate in collagen could ultimately have favorable effects. Thus, the rapidly increasing viable cell number in collagen gel, together with proven biocompatibility (Wakitani et

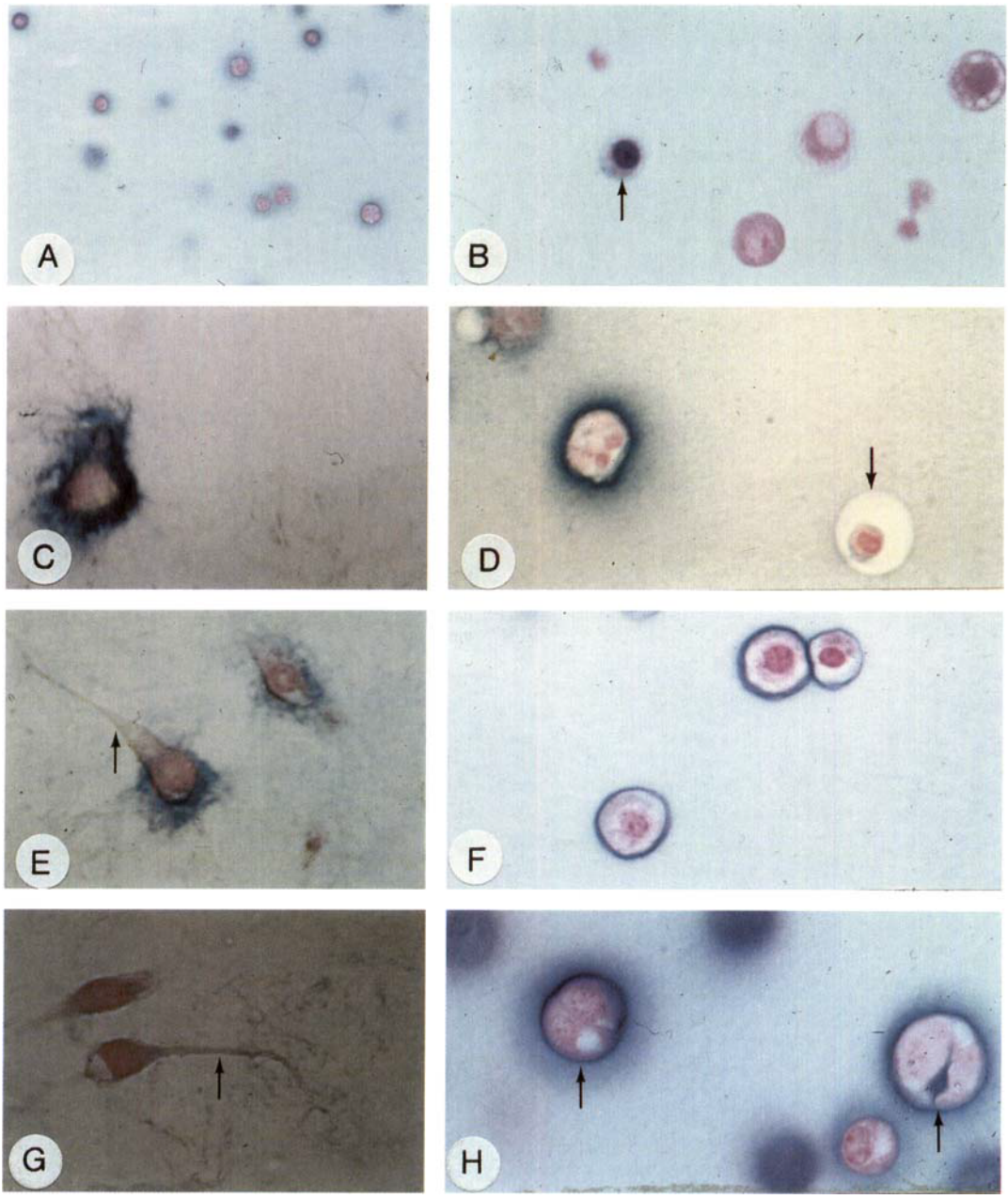

Figure 4. Histology of chondrocytes in collagen gel (C, E, G) and in alginate gel (A, B, D, F, H) directly after isolation (B), 2 (A), 3 (C, D), 6 (E, F) and 9 days (G, H) after isolation, respectively. Arrows in B and D point to necrotic cells. Note in E and G the large cytoplasmic extensions of the cells (arrows). In H, arrows point to small clusters of chondrocytes. A ×90, B-H ×500

al. 1989, Grande et al. 1989, Burgess and Goode 1992), are important advantages of collagen type I as a carrier for chondrocyte transplantation.

In accordance with previous work (Grandolfo et al. 1993, Häuselmann et al. 1994), no dedifferentiation of chondrocytes was noted in alginate gel throughout the culture period. There was a gradual decrease in the production of cartilage matrix around the cells in the alginate gel (Bentley and Greer 1971, Wakitani et al. 1994). However, matrix production, calculated per cell in alginate gel, increased rapidly with time and surpassed that in collagen gel on day 6. Cell death may have caused the initial low matrix production in alginate gel, owing to exposure to high Ca^{2+} concen-

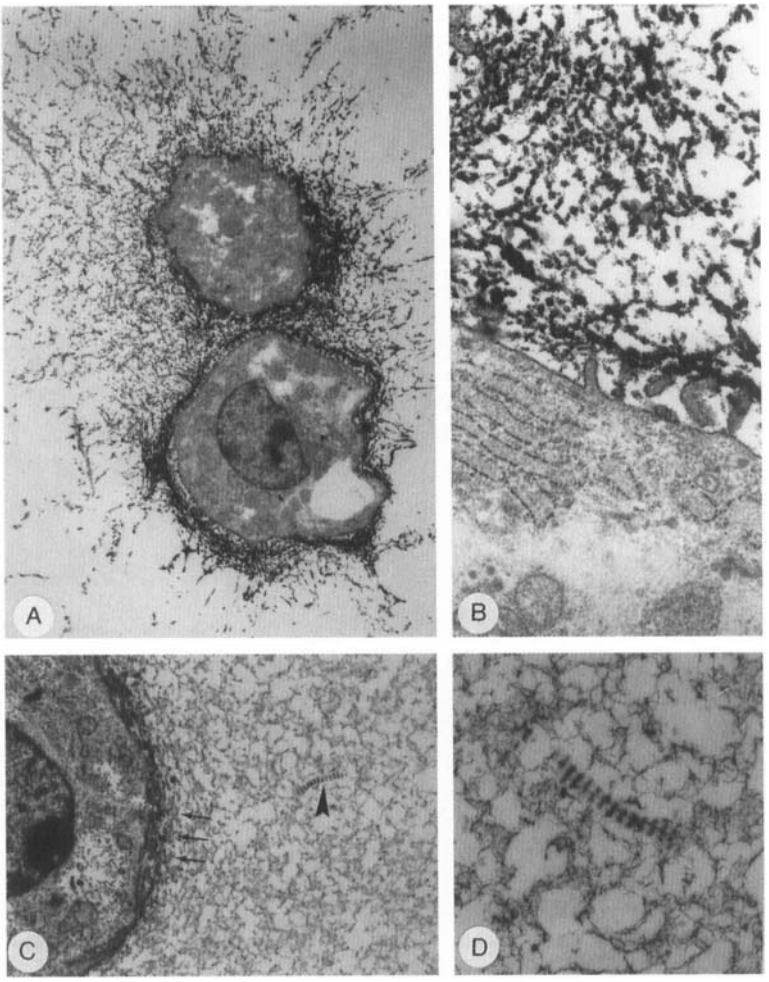

Figure 5. Electron microscopy of chondrocytes cultured in collagen gel and alginate.
A. 6 days of culturing in collagen gel. Chondrocytes produced a pericellular layer of matrix, without clear morphological characteristics. ×2500.
B. Enlargement of A. ×20,000.
C. Cell in alginate, 3 days after isolation. Pericellular matrix is accumulating (arrows). ×6000. Note banded structure at some distance from the surface of the cell (arrowhead).
D. Enlargement of C. ×20,000.

trations during gelation, especially as it occurred mainly during the first few days of culture. However, the diffusion rate of nutrients into the relatively dense porous structure of alginate gel could also be a limiting factor. The maintenance of typical chondrocyte morphology and the steady increase in matrix production indicate that alginate provides a good environment for chondrocyte cultures, but further in vitro studies are needed to investigate the biocompatibility of such an implant. Although not tested in this study, we believe that greater pericellular matrix formation will increase the mechanical strength of the implant, as has been observed in agarose cultures (Buschmann et al. 1992).

Chondrocytes could easily be cultured in combination with DBM, but no effect on matrix production was observed in either the collagen or alginate gels. We expect that positive effects of DBM will be found in vivo, because remodeling of the DBM fragments will result in the actual release of chondrogenic growth factors present. In vitro experiments (preliminary unpublished data) showed that the matrix production of chondrocytes in alginate can be stimulated by insulin-like growth factor and transforming growth factor-ß. The tendency for DBM to remodel in an articular cartilage defect and to become incorporated into the subchondral bone has already been demonstrated by Billings et al. (1991). Future animal

experiments may be able to establish whether a composite transplant of chondrocytes with DBM results in better fixation of the graft and in repair tissue which more closely resembles hyaline cartilage.

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