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Tissue Engineering of Articular Cartilage under the Influence of Collagen I/III Membranes and Low Oxygen Tension*

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ABSTRACT

The objective of this study was to study the matrix production and phenotype stability of articular chondrocytes cultured on collagen I/III membranes (CM) under the influence of low oxygen tension (Po_2). Primary bovine and osteoarthritic human chondrocytes were cultured for 2 weeks under 5–21% Po_2 on CM, in alginate, or as monolayers. Dedifferentiated cells were produced by 2-week monolayer culture under 21% Po_2. Collagen (Coll) type II and I expression was demonstrated immunohistochemically, by Western blotting (Coll II), and by semiquantitative RT-PCR; proteoglycan synthesis was demonstrated histochemically (toluidine blue); and biosynthetic activity was indicated by radiolabel incorporation ([^{3}H]proline and [^{35}S]sulfate). Bovine chondrocytes on CM showed an increase in Coll II expression and proteoglycan synthesis under low Po_2 conditions, whereas Coll I decreased. This oxygen-dependent phenotype-stabilizing effect was even more pronounced in alginate cultures. Biosynthesis of bovine and human chondrocytes was also increased by low Po_2, except for proline incorporation, which decreased in bovine CM cultures (low-oxygen effects were significantly higher in alginate than in CM cultures). Dedifferentiated chondrocytes reexpressed Coll II protein when cultured under low Po_2 on CM or in alginate only, but not under high Po_2 or in monolayer culture. We conclude that CM and, even more, alginate foster phenotype stability and cartilage-specific matrix production of bovine chondrocytes, especially when cultured under *in vivo*-like oxygen conditions.

INTRODUCTION

AUTOLOGOUS CHONDROCYTE TRANSPLANTATION (ACT) is a well-documented treatment for certain types of articular cartilage defects.[1] To cover the defect and to keep the injected cultivated chondrocytes in place, a periosteal graft is used. In addition, periosteum possesses chondrogenic potential, because of the presence of a cambial layer with mesenchymal stem cells and therefore its benefit in ACT is considered to be dual.[2–4] However, the quality of the periosteum differs among patients and is age dependent, and specific surgical training is required to retrieve the mesenchymal layer of the periosteum.[5,6] Furthermore, periosteum causes hypertrophy of the repair tissue in up to 28% of cases, requiring arthroscopy to remove the overgrowing tissue in some of these patients.[1] In a sheep study periosteal grafts induced strong bone production in the subchondral bone underneath the defect area, which is thought to promote the progression of cartilage degeneration.[7,8] Many researchers therefore try to exchange periosteum with a scaffold, or to create a matrix construct with cells to implant in the cartilage defect.

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A variety of different scaffolds for cartilage tissue engineering have been described: for example, polyglycolic acid and polylactic acid, fibrin, alginate, polyethylene oxide, self-assembling peptide hydrogels, or collagen type I gels, and collagen type I and II sponges.[9–15] Many of these scaffolds maintain differentiated chondrocytes and accumulate extracellular matrix *in vitro*, but so far most of them are not available for clinical application. One commercially available scaffold is acellular porcine collagen I/III membrane (Chondro-Gide; Geistlich, Wolhusen, Switzerland). It might be a promising biodegradable carrier for chondrocytes, because it has already been used clinically for the treatment of articular cartilage defects and there has been no bone-inducing potential described for this scaffold.[7,16] However, little is known about the influence of this membrane on the metabolic activity and phenotype stability of chondrocytes.

When chondrocytes are transferred from cell culture into a cartilage defect the cells will be exposed to native joint conditions, which generally are different from standard culture conditions. One aspect is the oxygen tension (Po_2), which has been described to be lower than 8% in avascular cartilage *in vivo* in contrast to 21% in cell culture incubators.[17] As a consequence, chondrocyte metabolism is adapted to these conditions.[18,19] Oxygen alters proliferation, matrix production, and phenotype stability of primary and dedifferentiated chondrocytes in monolayer and alginate culture systems.[20–23] In addition, expression of chondrocyte characteristics in mesenchymal cells is favored by low Po_2 and chondrocyte and organ cultures of cartilage exhibit properties typical of juvenile cartilage under these conditions.[24,25] Thus, it might be speculated that the metabolism of chondrocytes cultured on other scaffolds, such as collagen I/III membranes (CMs), might also be influenced by oxygen. For that reason we investigated the matrix production and phenotype stability of primary and dedifferentiated chondrocytes cultured on CMs under various Po_2 conditions in comparison with alginate cultures. Our data demonstrate that CMs foster articular chondrocyte matrix production and phenotype stability like alginate, especially under *in vivo*-like oxygen conditions, although this effect seems to be less pronounced in CMs in comparison with alginate.

MATERIALS AND METHODS

Isolation and cultivation of bovine and human chondrocytes

Articular cartilage samples were harvested from the articulatio tarsi transversa of adult cattle or from human femoral and tibial condyles, which were isolated during implantation of knee endoprotheses (Endo-Clinic, Hamburg, Germany). All samples were washed in Hanks' buffered salt solution (HBSS, containing penicillin–streptomycin [100 U/mL] and amphotericin [2.5 μg/mL]; Biochrom, Berlin, Germany), diced aseptically, and treated with a mixture of Pronase (32 U/mL; Roche, Mannheim, Germany) and hyaluronidase (2.3 KU/mL; Sigma, Deisenhofen, Germany) in HBSS for 1 h. After washing with HBSS with antibiotics (see above) extracellular collagen was digested with collagenase (Sigma) (918 U/mL in Ham's F-12 medium [Biochrom] with vitamin C [50 μg/mL; Sigma] and vitamin E [50 μM; Sigma]) for 6–8 h. The cell suspension was centrifuged (10 min at 400 $\times$ *g*) and the pellet was resuspended in Ham's F-12 medium with 10% fetal calf serum (FCS; Biochrom), antibiotics, and vitamins (see above) and filtered through a gauze filter (mesh size, 20 μm, Hydrobios, Kiel, Germany). Viable cells were counted, using a Neubauer chamber and the trypan blue exclusion method, and seeded as primary cultures on collagen I/III membranes or as monolayers with a density of 100,000 cells/cm^2, or transferred into an alginate gel (protocols described below). Medium was exchanged twice a week. In some cases human serum was isolated from the blood of patients and used for cell culture instead of FCS.

Dedifferentiated chondrocytes were produced by 2-week monolyer culture under 21% oxygen conditions. The dedifferentiated cells were then trypsinized (0.25% in phosphate-buffered saline [PBS]; Sigma) and dispersed (see above). To redifferentiate, the cells were seeded on collagen I/III membranes, in alginate, or as monolayers in culture wells.

Collagen I/III membrane and alginate cultures

Primary or dedifferentiated chondrocytes were seeded on the rough side of CMs (Chondro-Gide; a gift from Geistlich Biomaterials, Wolhusen, Switzerland) at a density of 100,000 cells/cm^2. Before use the CMs were washed several times with PBS. Alginate cultures were prepared by dispersion of isolated cells at a density of 10^7 cells/mL in sterile alginate solution (1.2% sodium alginate [Fluka, Deisenhofen, Germany] in 0.9% saline solution). Alginate beads (diameter, $\sim$2 mm) were made by dropping alginate–cell suspension through an injection needle (diameter, 0.9 mm) into $CaCl_2$ suspension (102 mM). The beads were cultured in Dulbecco's modified Eagle's medium (DMEM; Biochrom) containing 10% FCS, glutamine (2 mM), antibiotics, and vitamins (see above).

Cultivation under different oxygen tension

Cell cultures were kept under an atmosphere of 5% O_2, 5% CO_2, and 90% N_2 or 10% O_2, 5% CO_2, and 85% N_2 in an O_2- and CO_2-regulated incubator (BB 6220 O_2; Heraeus Instruments, Hanau, Germany) or under 21% O_2, 5% CO_2, and 74% N_2 in a CO_2-regulated incubator (Forma Scientific, Marietta, OH). For pH measurement

we equilibrated medium without cells under 5 and 21% O_2 and determined the pH with a pH electrode (WTW, Weilheim, Germany) after 8, 24, 48, and 72 h. No oxygen-dependent differences in medium pH were found.

Sectioning for histology

For paraffin embedding, cell-seeded CMs were fixed overnight with 4% paraformaldehyde (in PBS) and alginate beads were fixed overnight with 4% paraformaldehyde (in 29 mM $NaH_2PO_4 \cdot 1\ H_2O$, 28 mM Na_2HPO_4, 53 mM saccharose, and 10 mM $CaCl_2$; pH 7). CMs were washed with PBS and alginate beads were washed with cacodylate buffer (0.1 M cacodylate, 50 mM $BaCl_2$, and 0.005% eosin, pH 7.4). CMs were dehydrated with rising concentrations of ethanol and alginate beads were dehydrated with isopropanol (75, 96, and 100%). Both types of samples were transferred into xylol, and embedded in paraffin. Thin sections (7 μm) were made with a sled microtome (Leitz, Wetzlar, Germany) and spread out on slides coated with chrome alum gelatin (4.5% [w/v] gelatin, 300 mM chromium potassium sulfate, 12-hydrate).

Immunocytochemical staining of collagens

Paraffin sections (see above) were deparaffinated with xylol and transferred into distilled water, using decreasing concentrations of ethanol. They were treated with pepsin (3.9 kU/mL 0.5% acetic acid; Sigma) for 30 min and rinsed with Tris-buffered saline (TBS: 0.14 M NaCl in 20 mM Tris–HCl buffer, pH 7.4). Unspecific peroxidases were blocked by treatment with 0.6% H_2O_2 in methanol for 20 min and samples were rinsed with TBS and treated with primary antibody (collagen type II: monoclonal antibody [MAb] mouse anti-collagen type II, clone CII C1, [Developmental Studies Hybridoma Bank, Iowa City, IA], diluted 1:1000 in TBS; collagen type I: MAb mouse anti-collagen type I [C-2456; Sigma], diluted 1:1000 in TBS) for 1 h (controls were incubated with TBS without the first antibody). After rinsing with TBS the samples were treated for 30 min with the second antibody (MAb rabbit anti-mouse IgG, horseradish peroxidase [HRP] conjugated [P-0260; DakoCytomation, Hamburg, Germany], diluted 1:200 in TBS containing 1% bovine serum), rinsed again, and incubated for 30 min with the third antibody (MAb goat anti-rabbit IgG, HRP conjugated [P-0448; DakoCytomation], diluted 1:100 in TBS containing 1% bovine serum). The samples were stained with diaminobenzidine (DAB kit; Vector Laboratories, Burlingame, CA). Cell nuclei were counterstained with Meyer's hemalum (Merck, Darmstadt, Germany) and the stained samples were embedded with Aquatex (Merck).

Toluidine blue staining

Paraffin sections (see above) were deparaffinated with xylol and transferred into distilled water, using decreasing concentrations of ethanol. The sections were incubated for 6 min with toluidine blue solution (0.0714% toluidine blue [Merck], 0.0714% pyronin Y [Fluka, Buchs, Switzerland], and borax [0.143% disodium tetraborate [Merck]); washed with distilled water, 96% ethanol, 3× propanol (2 min each), and 3× xylol (15 min); and mounted on microscope slides with DePeX (Serva, Heidelberg, Germany).

[^{3}H] Proline and [^{35}S]sulfate incorporation/ DNA measurement

CM and alginate cultures were incubated overnight in culture medium (see above) containing [^{3}H]proline or [^{35}S]sulfate (10 μCi/mL each; Amersham Biosciences, Piscataway, NJ) and washed in PBS (for alginate, PBS containing 1 mM Ca^{2+}). Each sample was digested separately at 65°C overnight in 1 mL of papain solution (0.125 mg/mL [2.125 units/mL; Sigma]), 0.1 M Na2HPO$_4$, 0.01 M EDTA, pH 6.5). A 200 μL volume of each sample was added to 2 mL of scintillation fluid (Hydroluma; Mallinckrodt Baker, Phillipsburg, NJ) and measured with a scintillation counter (Wallac, Turku, Finland). A 500-μL volume of each sample was mixed with 500 μL of PBS and used to determine the DNA content. Samples and blanks (containing 1 mL of PBS) were treated for 15 s with an ultrasonic beam (Sonoplus gm70; Bandelin, Berlin, Germany). A 0.5-mL volume of RNase (5 KU/mL RNase/Pronase buffer [PBS diluted 1:5 with buffer (50 μM $MgCl_2 \cdot 6H_2O$ and 90 μM $CaCl_2 \cdot 6H_2O$)] and 0.5 mL of Pronase (0.7 U/mL RNase/Pronase buffer) were added and incubated for 30 min at 37°C. A 0.5-mL volume of ethidium bromide (25 μg/mL) was added and samples were incubated for 30 min and measured with a fluorometer (F2000; Hitachi, Tokyo, Japan) (λ_{ex}, 365 nm; (λ_{em}, 590 nm). DNA content was computed by comparison with a standard curve. Significance of differences was calculated by two-tailed Student t test (with significance at $p < 0.05$).

Western blot for collagen type II

CM or alginate cultures were incubated at 4°C for 24 h with 500 μL of 0.05 M acetic acid/cm^2 CM or 5 alginate beads including pepsin (0.1 mg/mL; Sigma). A 50-μL volume of a 10× TSB stock solution (1 M Tris, 2 M NaCl, and 50 mM $CaCl_2$, pH 8) and 50 μL of pancreatic elastase (1 mg/mL TSB; Sigma) were added and samples were incubated for 30 min at 37°C. The samples were treated with an ultrasonic beam and centrifuged for 10 min at 9000 × g. A 25 μL volume of collagen type II standard (0.4 and 0.6 μg; Sigma) or supernatant sample was mixed with 6 μL of sample buffer (RotiLoad 1; Roth, Karlsruhe, Germany), denatured for 5 min at 95°C, and loaded on a 4–12% gradient gel (NuPage; Invitrogen, Carlsbad, CA). Electrophoresis was started (NuPage sys-

tem [Invitrogen] and Power Pac 200 [Bio-Rad, Hercules, CA]) at 200 V and 110 mA/gel. The gel was then transferred onto blotting membrane (Protran; Schleicher & Schuell, Dassel, Germany) and the proteins were blotted for 5 h at 25 V and 80 mA, using a continuous buffer system (50 mM Tris, 380 mM glycine, 0.1% sodium dodecyl sulfate [SDS], 20% methanol) in a Bio-Rad Trans-Blot SD semidry transfer cell. The membrane was blocked overnight in blocking buffer (10% milk powder [w/v]) in TBST [20 mM Tris, 1 mM EDTA, 0.14 M NaCl, 0.1% Tween 20, pH 7.5]), incubated with anti-collagen type II antibody (CII C1 supernatant, [Developmental Studies Hybridoma Bank], diluted 1:20 in TBST) for 2 h, and washed with TBST. The second antibody (Dako-Cytomation P0260) was added (diluted 1:30,000 in TBST) for 1 h. The blot was rinsed and incubated for 1 h with the third antibody (DakoCytomation P0448, diluted 1:10,000 in TBST). Bands were visualized on film (Hyperfilm ECL; Amersham Biosciences), using an ECL Plus kit (Amersham Biosciences).

Reverse transcription-polymerase chain reaction

For RNA isolation 5 alginate beads per sample were dissolved in dissolving buffer and cells were pelleted by centrifugation (2 min). CM cultures (5 membranes of 1 cm^2/sample) were trypsinized for 8 min and centrifuged for 2 min in order to obtain a cell pellet. RNA was isolated from the cell pellets with a Qiagen (Chatsworth, CA) Rneasy minikit according to the manufacturer's instructions. Lysed samples were homogenized with QIAshredder spin columns (Qiagen). Isolated RNA was examined spectrophotometrically for quantity and quality (absorbance at 260 and 280 nm). cDNA was generated with a Qiagen OneStep reverse transcription-polymerase chain reaction (RT-PCR) kit according to the manufacturer's instructions. For PCR the following bovine primers were used (0.5 μM): collagen type II (sense, 5′-AAG AAA CAC ATC TGG TTT GGA GAA ACC-3′; antisense, 5′-ATG GGT GCA ATG TCA ATG ATG GG-3′; product size, 342 bp; annealing temperature, 59°C) and collagen type I (sense, 5′-GAT GGA GAC TTC TAC AGG GCT GAC-3′; antisense, 5′-CGA TGT CCA AAG GTG CAA TAT CAA GG-3′; product size, 712 bp; annealing temperature, 62°C). RT-PCR conditions were as follows: reverse transcription, 30 min at 50°C; initial activation step, 15 min at 95°C; three-step cycles (melting, 30s at 94°C; annealing, 60 s at primer-specific temperature [59–64°C, see above]; and a final extension step, 10 min at 72°C). Amplified products were separated on a 2% agarose gel, visualized fluorometrically, digitized, and quantified by comparison of band intensities. To avoid errors raised by RNA quantification in each individual sample, PCR products were compared with different primer products of the same RNA sample only. In addition, aliquots of every RT-PCR were taken after 15 and 17 cycles in order to find the earliest time point at which products of the various mRNAs show up in the gel.

RESULTS

Primary bovine chondrocytes

Primary bovine chondrocytes on collagen I/III membranes (CMs) and in alginate beads showed an oxygen-dependent deposition of Coll II protein in their pericellular matrix (Fig. 1). Coll II expression was increased under reduced oxygen tension (Po_2) in both cases, at the protein level (Figs. 1 and 2) and at the mRNA level (Figs. 3 and 4). Coll I expression as a marker of dedifferentiation was also found in both culture systems. But, in contrast to Coll II, expression of Coll I protein and mRNA was increased under higher Po_2 conditions (Figs. 1, 3, and 4).

Although CM and alginate cultures showed the same tendencies regarding oxygen-dependent expression of Coll II and I, there were differences between these two scaffolds. The ratio of Coll II to Col I mRNA products was 11- to 16-fold higher in the alginate system, indicating a stronger phenotype-stabilizing influence in comparison with CM (Fig. 4). Immunohistochemical stainings showed slightly lower collagen type I protein expression in the alginate system in comparison with the CM cultures (Fig. 1; the CM itself did not show any specific collagen type I immunostaining, indicating some loss of antigenicity). In the alginate system proline incorporation of primary chondrocytes was increased significantly, 1.35-fold, under low Po_2 conditions in comparison with standard culture conditions. In contrast, the protein biosynthetic activity of the cells was decreased (about 30%) in CM cultures (Fig. 6). In addition, proline incorporation was significantly lower in CM cultures under low oxygen conditions in comparison with alginate cultures.

Proteoglycans were deposited in the matrix of both CM (Fig. 1) and alginate cultures (not shown). But toluidine blue staining showed more intense purple color in the extracellular matrix of cultures kept under low Po_2. This was supported by sulfate radiolabeling, which was increased under low Po_2 conditions in both culture systems (Fig. 7; in alginate cultures sulfate incorporation was increased significantly, about 3.23-fold, under 5% Po_2, whereas in CM cultures the 1.22-fold increase was not significant). Again, sulfate incorporation was significantly lower in CM cultures under low oxygen conditions in comparison with alginate cultures.

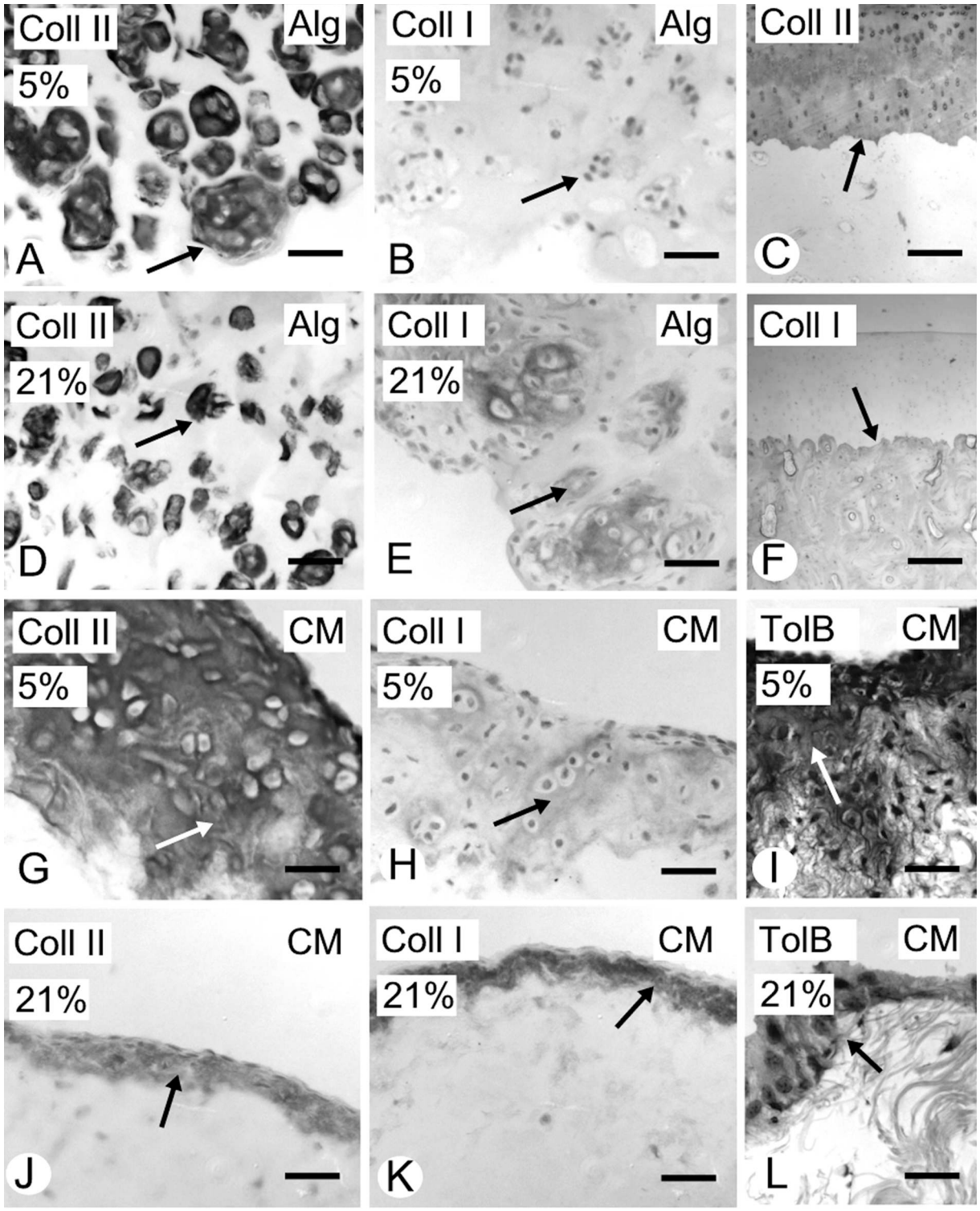

FIG. 1. (**A–L**) Bovine articular chondrocytes cultured for 14 days on collagen I/III membranes Coll II (CM) or in alginate (Alg) under various oxygen tensions (%). Coll II, immunohistochemical staining of collagen type II (arrows). Coll I, immunohistochemical staining of collagen type I (arrows). (**C** and **F**) Bovine articular cartilage and subchondral bone as positive controls for Coll I and II. (**I**) and (**L**) represent examples of toluidine blue staining (TolB). Scale bars: (**A, B, D, E,** and **G–L**) 50 μm; (**C** and **F**) 250 μm.

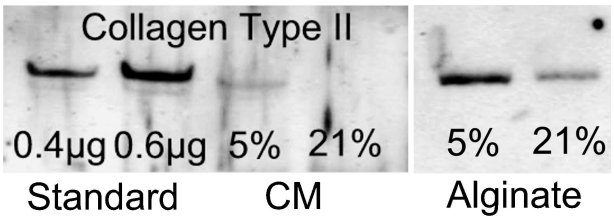

FIG. 2. Western blot for collagen type II from samples of bovine chondrocytes cultured for 14 days on collagen I/III membranes (CM) or in alginate under 5 and 21% oxygen tension. Lanes 1 and 2 show collagen type II standards.

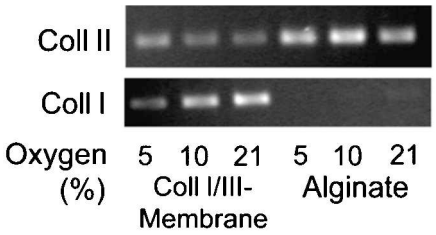

FIG. 3. One example from 3 independent experiments with representative RT-PCR products in agarose gel after 17 cycles. RNA isolation was from bovine chondrocytes after 14 days of culture under various oxygen tensions (%) in alginate or on collagen I/III membranes.

Dedifferentiated bovine chondrocytes

Chondrocytes were dedifferentiated in a 14-day monolayer culture under standard culture conditions. These cells were not able to reexpress Coll II protein under 5 or 21% oxygen conditions in a subsequent monolayer culture (not shown). However, on CM cultures (Fig. 5) and in alginate cultures (data not shown; see also Domm *et al.*)[21] the cells deposited new Coll II protein, but only when the cells were kept under low oxygen tension, indicating a redifferentiation of the chondrogenic phenotype only under these conditions. In alginate cultures proline incorporation by dedifferentiated cells was increased significantly, about 1.6-fold (Fig. 6) and sulfate incorporation was increased about 5-fold (Fig. 7) when oxygen was reduced. Nevertheless, in the CM system proline incorporation by dedifferentiated cells was decreased significantly, about 34%, under low oxygen conditions (Fig. 6) whereas sulfate incorporation was not significantly altered by oxygen (Fig. 7). In addition, proline and sulfate incorporation was significantly lower in CM cultures under low oxygen conditions in comparison with alginate cultures.

Human osteoarthritic chondrocytes

While human osteoarthritic chondrocytes were being cultured under various culture conditions it occurred to the authors that these cells were more complicated to handle than bovine cells. In monolayer culture cell adherence was low. Thus, in the following experiments human cells were used on CMs and in alginate only. Proline and sulfate incorporation of human chondrocytes was oxygen dependent (Figs. 6 and 7). Under low oxygen conditions sulfate incorporation in CM cultures was increased 2.15-fold and proline incorporation was increased 1.4-fold in comparison with 21% oxygen, whereas in alginate the increase was 1.5- and 1.25-fold, respectively. However, in histological observations no immunocytochemical staining of collagen type II was detectable after a 2-week culture period independent of oxygen and culture conditions (not shown). Biosynthetic activity was 3- to 7-fold lower in human cells than in bovine primary chondrocytes (Figs. 6 and 7). When human serum was used proline and sulfate incorporation were significantly increased in comparison with FCS (proline incorporation, 2.25-fold; sulfate incorporation, 1.5-fold; $p < 0.01$, $n = 4$). But still, when human serum was used human chondrocytes created a pericellular matrix that could not be visualized by immunocytochemical staining of Coll II and there were weak pericellular stainings with toluidine blue only (not shown).

DISCUSSION

Bovine primary chondrocytes and matrix production

Collagen expression of primary bovine chondrocytes was in principle similar in collagen I/III membrane (CM) and alginate cultures, even regarding the influence of different oxygen tensions (Po_2). In both types of culture

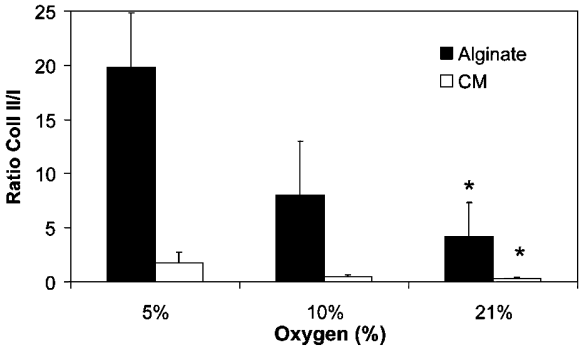

FIG. 4. Ratio of band intensities from RT-PCR products in agarose gels (mRNA for collagen type II versus collagen type I [Coll II/I]; see also Fig. 3). RNA isolation was from bovine chondrocytes after 14 days of culture under various oxygen tensions in alginate or on collagen I/III membranes (CM). Shown are mean values ± SD ($n = 3$ independent experiments). *$p <$ 0.05 versus corresponding 5% group.

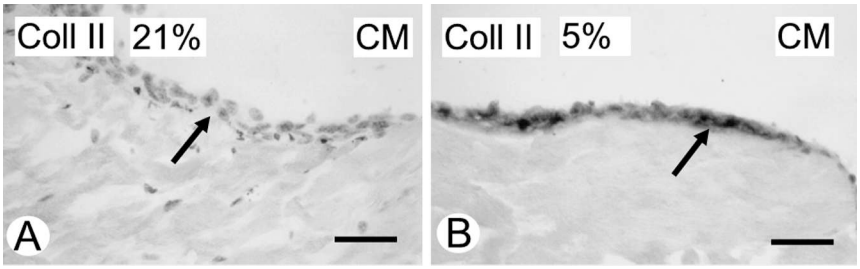

FIG. 5. Previously dedifferentiated chondrocytes reexpress collagen type II (Coll II: darker staining) after 2 weeks of culture on collagen I/III membranes (CM) under 5% (**B**) but not 21% (**A**) oxygen tension. Arrows show cell layers on the CM surface. Scale bars: 50 μm.

there was an increase in Coll II mRNA and protein expression under low oxygen conditions in comparison with Coll I expression, which was reduced at the same time. An increase in Coll II protein under low oxygen tension had already been described for monolayer and alginate cultures.[20,21] This supports the hypothesis that low oxygen tension favors cartilage-specific matrix protein deposition independent of the type of scaffold used. Immunocytochemically, we also found increased glycosaminoglycan (GAG) staining in CM and alginate cultures of bovine chondrocytes kept under low Po_2. Sulfate incorporation, on the other hand, was only slightly increased in CM cultures. However, Ysart and Mason found sulfate incorporation to be increased under 24% oxygen in comparison with 6% in cartilage explant cultures within the first 7 days of culture.[26] It might be speculated that native tissue responds differently than a tissue construct, where cells start to create a new matrix in comparison with normal turnover, which would be expected in explants. This needs to be investigated further. Nevertheless, on the basis of our data it can be concluded that in CM constructs and in alginate chondrocytes create a cartilage-like matrix, especially under *in vivo*-like oxygen conditions.

One important aspect of matrix production was the scaffold-dependent biosynthetic activity of the cells. Especially under low oxygen conditions, proline and sulfate incorporation of bovine cells was significantly higher in alginate cultures than in CM cultures. This could influence the maintenance or biomechanical properties of the matrices in the long-term.

Human primary chondrocytes and matrix production

Human cells were cultured on CMs. To our knowledge we have demonstrated for the first time that the biosynthetic activity of primary human articular chondrocytes is regulated by oxygen. Both sulfate and proline incorporation were increased under low oxygen conditions. However, in contrast to bovine cells, human chondrocytes did not produce a matrix that could be visualized

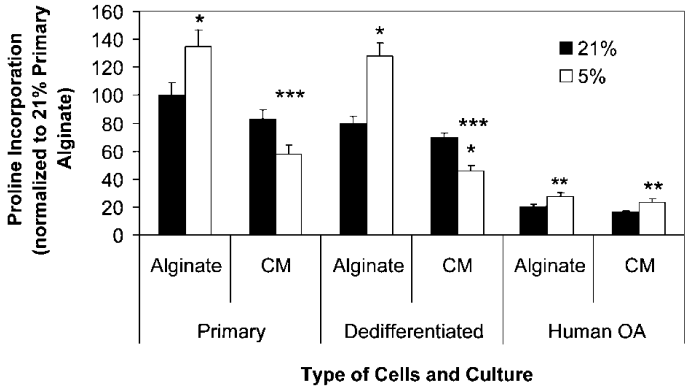

FIG. 6. Protein biosynthetic activity of articular chondrocytes on collagen I/III membranes (CM) or in alginate under the influence of 21 and 5% oxygen tension, measured by radiolabeled proline incorporation for 12 h on culture day 5. Shown are mean values ± SEM. CM: human, $n = 12$; primary bovine, $n = 20$; dedifferentiated bovine, $n = 16$. Alginate: human, $n = 12$; primary bovine, $n = 16$; dedifferentiated bovine, $n = 6$. *$p < 0.001$ and **$p < 0.01$ versus corresponding 21% group, ***$p < 0.05$ versus corresponding alginate group measured by two-tailed Student t test.

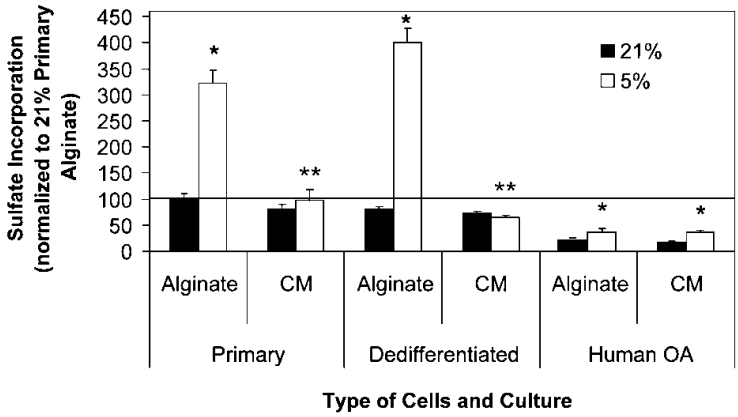

FIG. 7. Proteoglycan biosynthetic activity of articular chondrocytes on collagen I/III membranes (CM) or in alginate under the influence of 21 and 5% oxygen tension, measured by sulfate radiolabel incorporation for 12 h on culture day 5. Shown are mean values ± SEM. CM: human, $n = 11$; primary bovine, $n = 20$; dedifferentiated bovine, $n = 16$. Alginate: human, $n = 11$; primary bovine, $n = 16$; dedifferentiated bovine, $n = 12$. $*p < 0.001$ versus corresponding 21% group, $**p < 0.05$ versus corresponding alginate group measured by two-tailed Student t test.

by immunohistochemical staining of Coll II within 2 weeks of culture. This might be because the cells were from individuals 50 years of age and older, or because the state of disease of the donor influences matrix production. Our data regarding the biosynthetic activity of the cells showed up to 7-fold lower activity in comparison with bovine chondrocytes. Perhaps longer culture periods may be required to detect mature matrix. However, our finding raises questions concerning how effective these cells might be when transferred into a cartilage defect. We found a significant increase in both sulfate and proline incorporation when cells were cultured with autologous serum instead of FCS. Stimulation of proteoglycan synthesis and proliferation by human serum has already been described, and our data support the hypothesis that autologous serum seems to be superior for human cartilage tissue engineering with respect to matrix production.[27,28] However, in our study matrix production still was low in human cell cultures in comparison with the bovine system. It can be concluded that low oxygen tension and a three-dimensional scaffold alone still might not be sufficient stimulation to induce strong matrix production. Addition of growth factors might be necessary.

Phenotype stability of chondrocytes

When articular chondrocytes are cultured as monolayers the cells start to dedifferentiate. This is a process during which the cells become fibroblast-like and regain their ability to divide.[29,30] They lose their round phenotype, become spindle-shaped, and switch their collagen production from types II, IX, and XI to types I, III, and V.[31–33] Alginate, on the other hand, fosters production of cartilage-specific extracellular matrix components and promotes chondrogenesis in embryonal precursor cells or even a redifferentiation of dedifferentiated chondrocytes.[21,34–36] However, alginate might not be the first choice as a scaffold in cartilage tissue engineering because its biodegradability is low and its mechanical properties may be insufficient for cartilage defect treatment. Thus, CM might be an alternative. A stabilizing influence of CM on the chondrocyte phenotype has already been suggested by others, who found that sheep chondrocytes cultured on these membranes show more chondrocyte-like ultrastructural properties than do cells cultured on plastic surfaces.[37] In our study chondrocytes retain the ability to produce typical cartilage matrix molecules when cultured on CMs. In fact, CMs foster redifferentiation of dedifferentiated chondrocytes, in contrast to monolayer cultures, where new expression of Coll II was not observed. Thus, we speculate that the impact of CMs on the phenotypic stability of chondrocytes must be more chondrogenic than that of monolayer cultures. However, because the ratio of Coll II to Col I on the mRNA level seems to be lower in CM cultures in comparison with alginate, CMs still seem to be less chondrogenic than alginate. We found some immunohistochemical staining of Coll I in both primary CM and alginate cultures. Other authors have also described a weak production of Coll I by primary cells close to the surface of alginate beads after 2 weeks in culture or found minimal mRNA expression of the Coll I gene in alginate cultures.[34,38,39] Thus, it can be concluded that both scaffolds, CM and alginate, do not completely stabilize the chondrocyte phenotype.

Coll II reexpression by dedifferentiated chondrocytes on CMs revealed strong oxygen-dependent differences.

Under 21% oxygen no Coll II protein was detected, which leads to the conclusion that CM alone is not a sufficient stimulus for new reexpression of collagen type II protein. However, a combination of CM and low Po_2 seems to present a strong stimulus for redifferentiation, because under these conditions a majority of the cells showed strong Coll II production. Similar results had already been described for the alginate system.[21,22] In contrast, in monolayer cultures no reexpression of collagen type II could be measured even under low Po_2. This supports our conclusion that CMs and, even more, alginate scaffolds are more chondrogenic than monolayer cultures, and that more than one parameter (scaffold plus low oxygen tension) might be necessary to induce redifferentiation of dedifferentiated cells.

In conclusion, our study suggests that CMs can be considered scaffolds that allow primary articular chondrocytes to produce cartilage-like extracellular matrix components, stabilize the cellular phenotype, and promote redifferentiation of dedifferentiated cells, especially when exposed to *in vivo*-like oxygen conditions. Nevertheless, these properties still seem to be less pronounced than in the alginate system.

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