Cultivation of porcine cells from the nucleus pulposus in a fibrin/hyaluronic acid matrix

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ABSTRACT – Use of multiplied viable cells from the nucleus pulposus in altered discs, following in vitro cultivation, may be a promising therapy for degenerative disc disease.

Up till now, alginate has been used as a three-dimensional cell carrier to cultivate nucleus pulposus cells. However, the biocompatibility of the alginate, which depends on the composition and purity of alginate materials used, has not been considered for in vivo application so far. In this study, we cultured porcine cells from the nucleus pulposus in a mixture of fibrin and hyaluronic acid (HA). The DNA content and proteoglycan synthesis were compared to those measured in an alginate matrix. Although the increase in DNA content was 2.5-fold higher in the alginate culture after 3 weeks, the proteoglycan synthesis in relation to the DNA content was significantly higher in the fibrin/HA matrix.

We found that the fibrin/hyaluronic acid matrix can be used as a substrate for in vitro cultivation.

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Diseases of the intervertebral disc are characterized by changes in the extracellular matrix components that affect the mechanical function of the tissue (Duance et al. 1998). The inability of cells in the nucleus pulposus to regenerate causes the failure of an appropriate extracellular matrix (Leivseth 1999).

Therefore, new strategies aim to isolate a few nucleus pulposus cells to make them multiply and culture an increased number of cells in a substrate that promotes extracellular matrix production (Maldonado and Oegema 1992, Chelberg et al. 1995, Chiba et al. 1997). There is an ongoing discussion on the composition of the alginates used as to their biocompatibility. Most reports support the use of alginate with high guluronic acid content for in vivo studies while others suggest that alginate biocompatibility is independent of the mannuronic acid to guluronic acid residue ratio, but depends rather on the purity of alginate materials used (Otterlei et al. 1991, De Vos et al. 1997, Kulseng et al. 1999). However, the alginate system has been used in a patient for pancreatic islet transplantation (Soon-Shiong et al. 1994)

Our study aimed to test a new biodegradable and biocompatible matrix for cultivating nucleus pulposus cells, while preserving their phenotypical stability, and to compare the synthesis rate of specific macromolecules with that of the alginate system. We used a matrix composed of hyaluronic acid and fibrin, since much clinical experience has been gained with both of them (Shigeno et al. 1995, Akizuki et al. 1997, Harris et al. 1999).

Material and methods

Cell isolation

The lumbar sections of the spine from 5 male pigs aged 3 months were taken by en-bloc resection. Under a laminar flow hood, the intervertebral discs were dissected from the spine. Regions of anulus fibrosus and nucleus pulposus were visually identified. The separated nucleus pulposus of each pig was pooled in Leibovitz's L-15 medium (Biochrom, Berlin, Germany). For subsequent digestion, the enzymes were dissolved in a mixture (1:1) of Dulbecco's modified Eagle medium (DMEM; Serva, Heidelberg, Germany) and

Ham's F-12 medium (Biochrom, Berlin, Germany) supplemented with 5% fetal calf serum (FCS; Biochrom, Berlin, Germany). The tissue was first incubated with 0.4% pronase E (Serva, Heidelberg, Germany) for 1 hour, followed by overnight digestion with 0.02% collagenase (Serva, Heidelberg, Germany) at 37° with stirring. Following removal of tissue debris by filtering through a nylon mesh (100 μm), the isolated cells were rinsed three times in phosphate buffered saline (PBS). After determination of viability in a trypan blue exclusion test and cell counting using a hemocytometer, modified by Neubauer, the cells were seeded into a culture dish (100 × 20 mm).

Culture in monolayer

After enzymatic digestion, 2.4×10^6 (SD 0.19×10^6) cells were isolated from the tissue. The isolated nucleus pulposus cells were seeded in a monolayer culture at a density of 1.6×10^5 cells/cm^2. Cells were fed every 2 days with DMEM-Ham's F-12 supplemented with 10% FCS, streptomycin (100 μg/mL), penicillin G (100 IU/mL) and amphotericin (1 μg/mL). The cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

Culture in three-dimensional matrices

After 2 passages in monolayer culture, the cells of the P2 culture were embedded in a three-dimensional matrix. They were suspended in sterile saline solution containing either 2.25% fibrinogen (Beriplast, Centeon, Marburg, Germany) mixed with 0.33% hyaluronic acid (Ostenil, Chemedica, München, Germany) or 1.2% alginate (Sigma, St. Louis, MO, USA) at a density of 1×10^6 cells/mL. Without formation of air bubbles, 300 μL of the cell suspension were carefully added to the bottom of an insert with a microporous membrane (0.4 μm; Bibby Sterilin, Stone, England). The inserts were placed in 24 well plates. The alginate was polymerized by adding 100 μL of 102 mM $CaCl_2$ solution to each insert (10 min). Using 150 μL of a thrombin solution, the fibrinogen suspension mixed with HA was transformed into a gel-like condition. The polymerization time was delayed by diluting the thrombin solution with 40 mM $CaCl_2$ in a ratio of 1:3 (20 min). Following polymerization of the gels the polymerization solution was thoroughly aspirated off and the inserts rinsed with DMEM-Ham's F-12. Then the cultures were incubated with fresh medium supplemented with 10% FCS at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for 21 days. The medium was changed every day (1500 μL/well). Degradation of fibrin during the incubation was inhibited by adding of 250 KIE/mL of aprotinin (Trasylol, Bayer, Leverkusen, Germany) to the culture medium.

Proteoglycan synthesis

At 7, 14 and 21days, the cultures were incubated with (^{35}S) sulfate-labeled medium (Amersham Buchler, Braunschweig, Germany) for 24 hours (17 μCi/mL [0.63 MBq/mL]). Following lyophilization, gels were homogenized in 2 M sodium chloride. Aliquots were taken from each homogenate for simultaneous determination of the DNA content. To analyze the total proteoglycan synthesis, the homogenates were extracted overnight with 4 M guanidine HCl (GuHCL) buffered with 0.05 M sodium acetate (pH 5.8) and containing the following proteinase inhibitors: 0.01 M ethylenediamine tetra-acetate (EDTA), 0.1 M 6-aminohexanoic acid, 5 mM benzamidine HCl, 0.01 M N-ethylmaleimide (NEM) and 0.5 mM phenylmethyl-sulfonyl fluoride (PMSF). The extracts were eluted through a Sephadex G 25 (Pharmacia, Uppsala, Sweden) column (30 × 73 mm) with guanidinium hydrochloride. Radioactivity of the macromolecular fraction (first peak) was measured as dpm (disintegrations per minute) with liquid scintillation counting (LS 6000SC; Beckman, Fullerton, CA, USA).

DNA content

The determination of DNA content was done fluorometrically, as described by Labarca and Paigen (1980). At days 1, 7, 14 and 21, the gels were lyophilized and homogenized, as mentioned above. Following centrifugation, the supernatant was mixed with the fluorochrome bisbenzimide H 33258 (Serva, Heidelberg, Germany). The measurements of fluorescence were carried out in a spectrofluorometric detector (821-FP; Jasco, Hachioji City, Japan).

Hyaluronic acid content of the composite fibrin gel

The HA content of the growth medium of the mixed fibrin/HA cultures was determined by the thiobarbituric acid method (Jourdian et al. 1979). Briefly, 50 µL of growth medium were incubated with 50 µL 0.04 M sodium acetate buffer (pH 5.2) and 50 µl hyaluronidase (10^5 units/L; Calbiochem-Novabiochem, La Jolla, CA, USA) in 0.4 M sodium acetate buffer (pH 5.2) at 60° for 3.5 h. The enzyme specifically cleaves hyaluronan to unsaturated oligosaccharides which were quantified by a reaction with thiobarbituric acid and spectrophotometric analysis. The loss of HA from the gel corresponded to the HA content in the growth medium.

Histochemical and immunohistochemical staining

For light microscopy, gels were embedded in O.C.T. Compound (Tissue-Tek, Sakura Finetek, Torrance, CA, USA) and stored in liquid nitrogen at –140°C until required. By using standard histological techniques, serial sections of 4–6 µm were cut on a cryostat. Staining with hematoxylin and eosin (HE) and alcian blue at pH 1.7 was performed to assess cell distribution and matrix staining. The alcian blue staining was used to detect of proteoglycans.

For immunohistochemical analysis, the cryosections were mounted on glass slides pretreated with 3-aminopropyltriethoxysilane (Sigma, St. Louis, MO, USA). For proof of collagen II, the slides were incubated with rabbit polyclonal antibodies to collagen II (Chemicon, Hofheim, Germany; 1:200 1%BSA/PBS). Before incubation with the primary antibody to chondroitin sulfate proteoglycan, the cryosections were digested with chondroitinase ABC (Sigma, St. Louis, MO, USA). Then the slides were incubated with rabbit polyclonal antibodies to chondroitin sulfate proteoglycan (Biogenesis, New Fields, England; 1:500 1%BSA/PBS). The staining was visualized with a horseradish peroxidase-conjugated goat-anti-rabbit antibody and the 3-amino-9-ethylcarbazol substrate system (DAKO-Diagnostika, Hamburg, Germany).

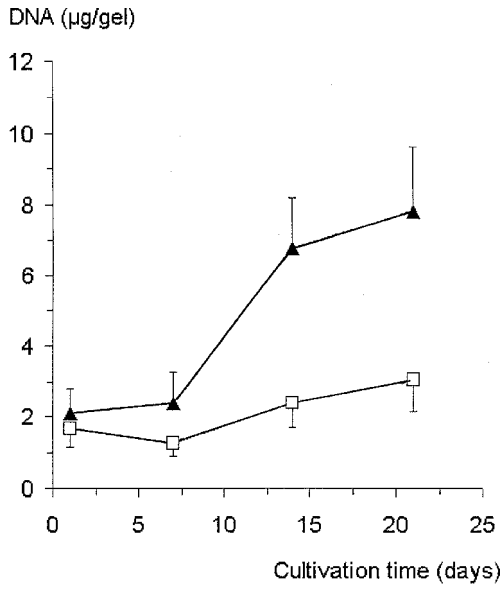

Figure 1. DNA content of the alginate and fibrin/HA carriers measured at days 1, 7, 14 and 21. Data are presented as mean ± SEM (n 5) (▲ alginate, ■ fibrin/HA).

Statistics

The experiments to determine DNA content and proteoglycan synthesis were performed 5 times. Each experiment group consisted of 2 specimens from each pig. Statistical analysis of the results to determine DNA content and proteoglycan synthesis was done using the Kruskal-Wallis test for unrelated samples to find differences between the alginate and the fibrin/HA matrix. The level of significance was $p < 0.05$.

Results

Cell number and viability

After enzymatic tissue digestion, a vitality rate of over 95% was established regarding the isolated cells (2.4×10^6 (SD 0.19×10^6) cells). The vitality of the cells in confluent monolayer cultures was over 90%. After 14 days of incubation in monolayer culture, a confluent cell aggregate had developed. A sufficient number of cells for cultivation in the three-dimensional matrix was obtained after two passages (11.5×10^6 (SD 0.27×10^6) cells).

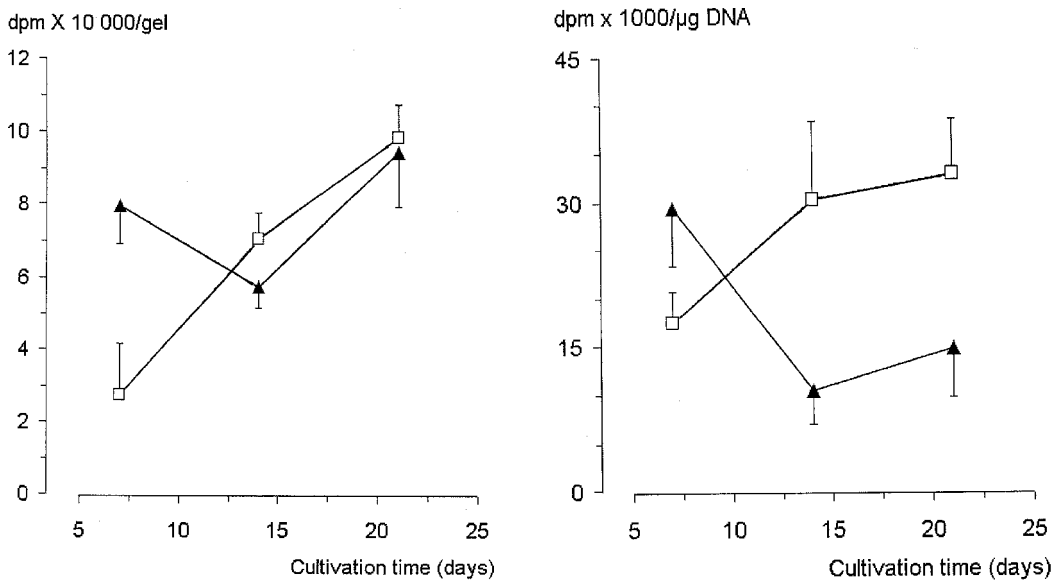

Figure 2. Proteoglycan synthesis of three-dimensionally cultivated nucleus pulposus cells in alginate and fibrin/HA carriers (left) measured at days 7, 14 and 21(▲ alginate, ■ fibrin/HA)and (right) referred to DNA content at days 7, 14 and 21 (▲ alginate, ■ fibrin/ HA). Data are presented as mean ± SEM (n 5).

DNA content

DNA content, which characterizes the proliferation capacity in the three-dimensional matrix, increased in both constructs over the entire course, whereby a steady state was not reached until the 21st day. While the DNA content in the fibrin-HA gel had approximately doubled after 21 days in culture, it had quadrupled in the alginate matrix in the same period. The DNA content on days 14 and 21 had significantly increased in the alginate gel, as compared to the fibrin-HA construct ($p = 0.03$) (Figure 1).

Proteoglycan synthesis

An increase in proteoglycan synthesis was found in the alginate and fibrin-HA matrices during three-dimensional cultivation. After 21 days, the amounts of newly synthesized proteoglycan were roughly equal in both cell matrices. Proteoglycan synthesis in relation to the DNA content, as an expression of synthesis activity of the individual cells, was significantly higher in the fibrin matrix than in the alginate matrix on days 14 and 21 ($p = 0.02$). 3 weeks later, the relative proteoglycan synthesis had doubled in the fibrin-HA matrix, which had a DNA content 2.5 times lower than that of the alginate matrix (Figure 2).

Histochemical and immunohistochemical staining

Histochemical and immunohistochemical staining proved that three-dimensionally cultivated nucleus pulposus cells create a pericellular matrix composed of both newly synthesized proteoglycans and collagen type II. As in the quantitative analysis, the immunohistochemical studies of the specimens showed an increasing pericellular accumulation throughout the duration of cultivation as an expression of increasing extracellular matrix component synthesis in both the alginate and fibrin/ HA matrices (Figures 3 and 4). The histochemical staining by alcian blue confirms these results, whereby an intense pericellular staining of the proteoglycans was observed, particularly in the fibrin/HA gel (Figure 5).

Hyaluronic acid retention in the gels

Approximately 50% of the HA initially injected had diffused out of the gel after 1 week. Over the further course, the composition of the matrix construct stabilized, and a steady state of 45% of the original hyaluronic acid amount was obtained at the end of cultivation (Figure 6).

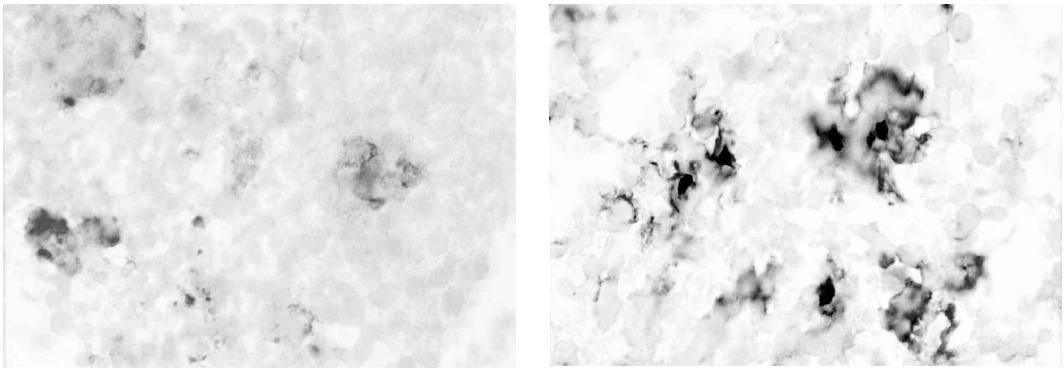

Figure 3. Immunolocalization of the pericellular accumulated collagen type II in sections of the fibrin/hyaluronic matrix at day 7 (left) and at day 21 (right) (×1000).

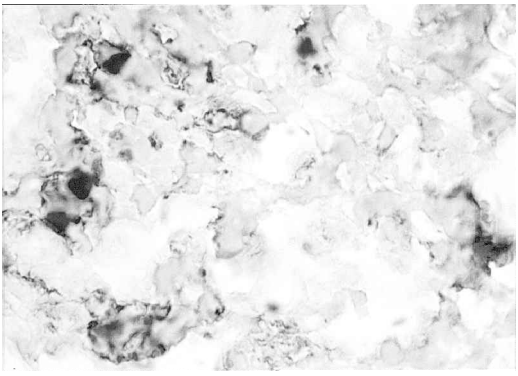

Figure 4. Immunolocalization of the pericellular accumulated proteoglycans in sections of the fibrin/hyaluronic matrix at day 21 (×1000).

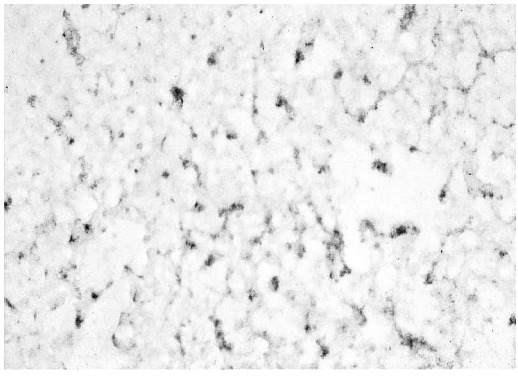

Figure 5. Staining by alcian blue of the pericellular accumulated proteoglycans in a fibrin/hyaluronic matrix at day 21 (x 200).

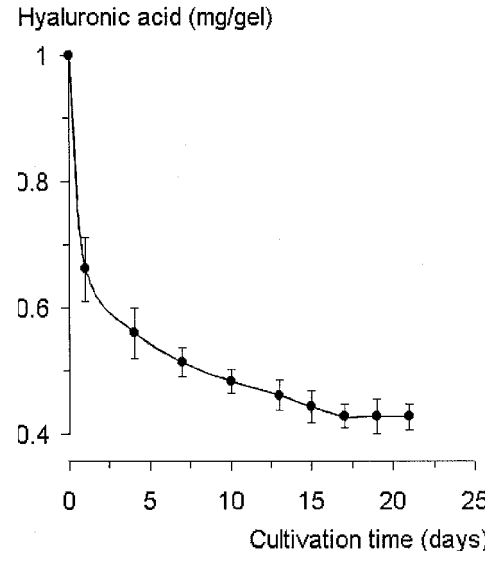

Figure 6. Retention of hyaluronic acid in the fibrin/HA carrier throughout three-dimensional cultivation of nucleus pulposus cells. Data are presented as mean ± SEM (n 5).

Discussion

Cartilage cells require a three-dimensional matrix to maintain their phenotypical stability; cultivation in monolayer cultures leads to dedifferentiation within a short time (Benya and Shaffer 1982, Bonaventure et al. 1994, Häuselmann et al. 1994). A very similar functional behavior has been shown for intervertebral disc cells synthesizing matrix-specific macromolecules (Gruber et al. 1997). The matrix substances alginate and agarose commonly used for culture may have an immunogenic potential which could limit their in vivo use (Maldonado and Oegema 1992, Chelberg et al. 1995, Gruber et al 1997, Chiba et al. 1997, Aguiar et al. 1999). Therefore, we assessed the cultivation and proliferation of nucleus pulposus cells in a biodegradable substrate made of fibrin and hyaluronic acid.

Homminga et al. (1993) found that articular chondrocytes maintain their characteristic morphology during short-term culturing in fibrin glue. We supplemented the growth medium with an anti-fibrinolytic agent, thus preventing the progressive disintegration of the fibrin. By adding HA, a typical matrix macromolecule is integrated into the three-dimensional cell carrier. Cells can adhere to the macromolecule via the CD-44 receptor (Aruffo et al. 1990) thereby directly affecting essential cellular functions and cytoskeletal structure. Hyaluronic acid thus prevents the loss of phenotype in chondrocyte cultures and increases proteoglycan synthesis, even when degenerative changes have already begun (Larsen et al. 1992). Recently, Lindenhayn et al. (1999) used a matrix system consisting of alginate, hyaluronic acid and fibrin to cultivate chondrocytes. The substantial loss of hyaluronic acid from the culture system resulting from the structural characteristics of this linear macromolecule was reduced by the combination with fibrin, as shown in that report. We suppose that the decrease in hyaluronic acid release after an initial drop-off in our study is due to an increasing stability with strong integration of the exogenous HA in the matrix construct and a steady state between loss of exogenous HA and synthesis of endogenous HA. This conclusion must be confirmed by further studies.

The increase in proteoglycan synthesis during cultivation was verifiable in both the alginate and the fibrin/hyaluronic acid gel. The diminished synthesis of proteoglycan in the fibrin/HA matrix, compared to the alginate matrix after 7 days is attributed to the inhibition of synthesis by higher pericellular concentrations of HA and its oligosaccharide derivatives (Wiebkin and Muir 1975, Knudson 1993). Later, the fibrin/HA construct stabilized at a reduced HA concentration level due to diffusion. At this point, synthesis activity rose to 3 times that of the alginate gel. The increased rate of proliferation noted in alginate gels is accompanied by a reduction in matrix synthesis, which suggests at least a temporary reduction in the number of differentiated cells.

This marked proliferation may be caused by low cell density (1×10^6 cells/mL suspension). Maroudas et al. (1975) defined a cell density of 4×10^6 cells/mL for the nucleus pulposus of human specimens. Since this method may be of clinical value, we chose this low cell density in our experiments because only small amounts of cells are available for in vitro cultivation. Furthermore, prolonged in vitro expansion of the few available cells may reduce their functional potential, although Gruber et al. (1997) found that the duration of cell proliferation in monolayer culture has no effect on the synthesis activity of cells in the subsequent three-dimensional cell culture.

The clinical approach based on these studies may involve 3 stages: (1) the isolation of suitable autologous cells, (2) in vitro culture and establishment of an injectable cell-matrix compound and (3) reimplantation of the newly formed nucleus pulposus. Clinical application would seen to make sense in young patients having mild-to-moderate disorders of disc structure. Degenerative changes in the intervertebral discs can now be recognized earlier than ever using MRI. Antoniou et al. (1998) presented the first clear evidence that quantitative magnetic resonance analysis reflects not only the disc matrix composition, but also its structural integrity.

These cell-based therapies could also be used in the adjacent segments of fused vertebral bodies and to improve disc function after dorsal stabilization with dynamic systems.

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