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Long-term maintenance of human articular cartilage in culture for biomaterial testing

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Abstract

Cartilage is a tissue that derives its unique mechanical and biological properties from the combination of relatively few cells and a large amount of a complex extracellular matrix. Furthermore, cartilage tissue is comparatively slow to respond to changes or harmful influences. To date, the optimal generation and long-term maintenance of cultured human articular cartilage for in vitro testing of biomaterials, poses an experimental difficulty. Experiments using cultured isolated chondrocytes in combination with scaffolds often fail to yield results comparable to the in-vivo situation. Consequently, our aim was to develop a culture method that allows in vitro maintenance of human hyaline cartilage explants in an optimal quality over an extended period of time. Such a culture could, for example, be used to determine the long-term effect of a new scaffold on intact cartilage, as an in vitro model for repair processes and to investigate biomaterial integration.

In this study we compared conventional static cultures with and without serum supplementation to a serum-free perfusion culture for the ability to maintain human articular cartilage explants in a morphologically intact and differentiated state over an extended period of time of up to 56 days. Results were evaluated and compared by morphological, histochemical and immunohistochemical methods.

The experiments showed that short-term maintenance of cartilage in a differentiated state for up to 14 days is possible under all culture conditions tested. However, best long-term culture results for up to 56 days were obtained with perfusion culture under serum-free conditions. Such a perfusion culture system can be used to perform biocompatabilty tests in vitro by long-term coculture of biomaterial and intact human articular cartilage.

Keywords: Biocompatability; Bioreactor; In vitro test; Cell culture; Human articular cartilage; Tissue Culture; Perfusion; Differentiation

1. Introduction

Hyaline cartilage has a very limited capacity for intrinsic functional repair [1]. Small chondral defects have been reported to regenerate by cell migration [2], but untreated large full-thickness defects in most cases progress to osteoarthritis with an eventual need for total knee replacement surgery [3,4]. Surgical and biological attempts have been made to induce significant and durable repair response in cartilage injuries. Such treatments include attempts to recruit progenitor cells from the bone marrow by penetration of the subchondral bone [5,6] or transplantation of osteochondral grafts, periosteum or perichondrium [7,8]. The therapies show acceptable short-term results but the repair tissue is fibrous and long-term data are usually unfavorable. The use of cultured cells in the autologous chondrocyte transplantation (ACT) technique is a promising

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alternative technique [9]. Autologous chondrocytes are expanded in vitro and injected under a periosteal flap sewed onto the defect. As shown by a large number of treated patients, ACT delivers good to excellent long-term clinical results well correlated with the formation of hyaline-like repair tissue within the injury site [10]. However, the method could be considerably improved using an advanced biomaterial instead of the periostal flap [11]. The procedure could generally be simplified and the open joint surgery could be replaced by a much safer arthroscopic operation [12,13]. Therefore, a number of flexible biomaterials needs to be evaluated for such purposes [14–17].

In order to test the biocompatibility of scaffolds and membranes that should be used within the joint in direct contact with articular cartilage, a good in vitro model will be necessary [18]. Such a model would have to allow long-term culture experiments because cartilage tissue can take weeks or months to show detectable pathology when exposed to harmful influences [19,20]. Long-term studies could also be valuable to investigate the so-far unknown underlying mechanisms of regeneration. Animal studies can yield very good insight [21] but the systems are highly complex and experimental parameters are difficult to control. As the transferability of findings from animal experiments to human tissue is problematic [22] in vitro models using human tissue should be used to complement and refine the results.

Consequently, our aim was to develop an in vitro model based on a tissue-specific culture that allows long-term maintenance of highly differentiated pieces of human cartilage for experimental purposes. In the present study, conventional static culture techniques were compared to a perfusion culture with respect to the ability to maintain human articular cartilage explants in a morphologically intact and highly differentiated state over short, intermediate and extended periods of time. All cultured explants were evaluated by a set of histochemical, morphological and immunohistochemical methods.

2. Materials and methods

2.1. Preparation of explants

Intact and healthy human articular cartilage was obtained from the femoral trochlear region of patients (four females/ two males; aged 21–39) undergoing patella regrooving operations. In this type of operation, a fairly large amount of healthy cartilage is removed. The patients had no history of cartilage damage or degeneration. Sample collection was performed with patient's consent under the ethical approval S040–01 of the Ethical committee of the medical faculty of Gothenburg University.

Pieces of cartilage were transported in phosphate buffered saline (PBS) from the surgical operating room and transferred to DMEM/F12 medium (Gibco BRL Life Technologies, Germany) immediately. A stainless steel punch with an inner diameter of 3 mm (Miltex Instruments, PA, USA) was used to punch out full thickness cylindrical explants of 3 mm diameter and 3 mm length (Fig. 1a). The average weight of these explants was 90 mg. Subchondral bone was not included in the preparation because it was not present in all material obtained from operations and because it would make sectioning of the samples much more difficult. Fresh explants from all six patients were processed and examined immediately to serve as controls.

2.2. Static culture

Static culture of the explants was performed in 12 well tissue culture plates (Becton Dickinson, Germany). Explants were placed into 1 ml of culture medium, each in separate wells of the culture plate. The three different culture media used were DMEM/F12 (Gibco BRL Life Technologies, Germany) + 50 μg/ml ascorbic acid + 50 μg/ml gentamycin + 2 μg/ml fungizone (Sigma, Germany) as serum-free base medium. Base medium supplemented with 10% fetal calf serum (FCS, Gibco BRL Life Technologies, Germany), and base medium supplemented with 10% autologous human serum (HS). Culture was performed in a humidified incubator (Haereus, Germany) at 37 °C. Culture medium was renewed every 3–4 days. Six individual samples were cultured in parallel in each medium for a period of 14, 28, 42 and 56 days.

2.3. Perfusion culture

Perfusion culture of the explants was performed in specially designed culture containers (Minucells and Minutissue, Germany) that allow continuous medium perfusion (Fig. 1b and c). The containers were placed on a heating plate (Medax, Germany) to maintain a constant temperature of 37 °C. Serum-free DMEM/F12 + 50 μg/ml ascorbic acid + 50 μg/ml gentamycin + 2 μg/ml fungizone + 35 mM HEPES (Sigma, Germany) was continuously perfused through the container using an IPC-N 8 peristaltic pump (Ismatec, Germany) at a rate of 1 ml/h. Used medium was not reperfused but collected as waste. The addition of 35 mM HEPES buffer was necessary to maintain the medium at a physiological pH of 7.4 under room atmosphere (0.3% CO_2). Six individual samples were cultured in parallel in each medium for a period of 14, 28, 42 and 56 days.

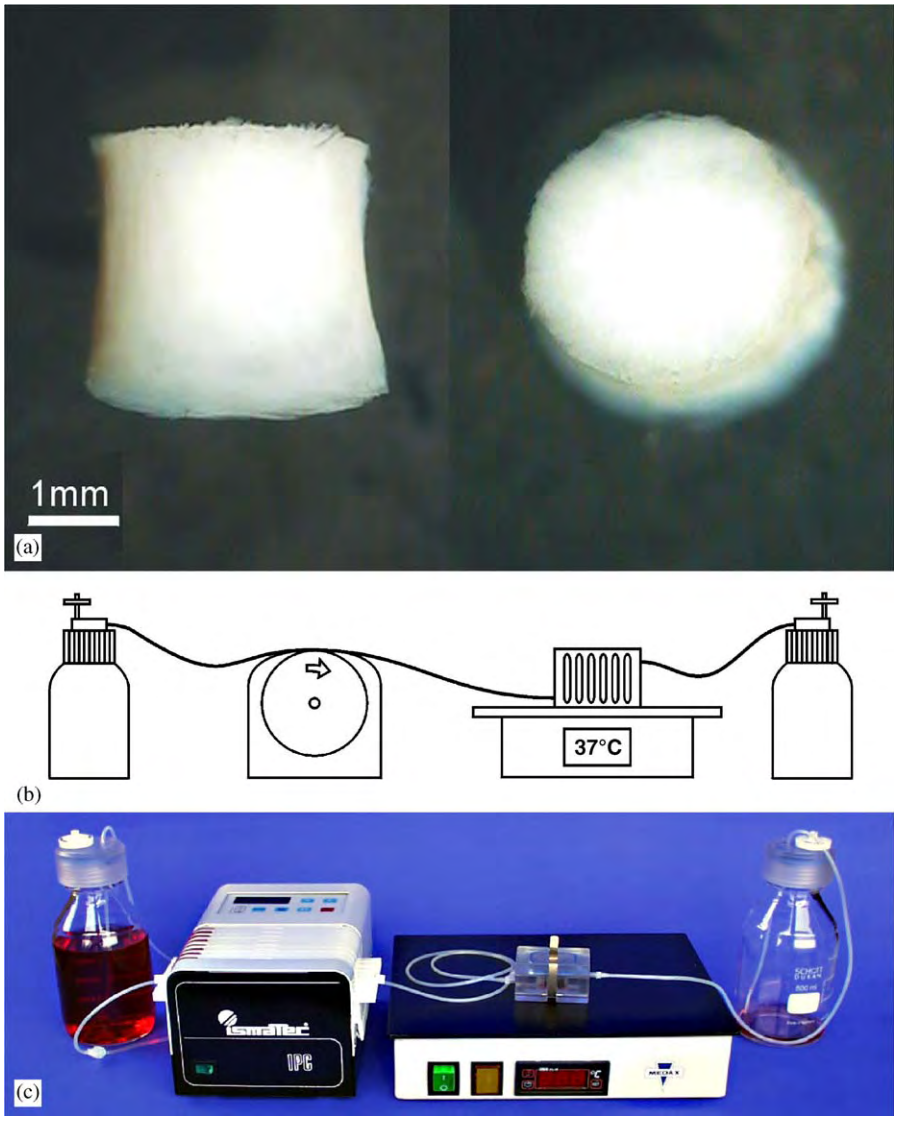

Fig. 1. (a) Human articular cartilage explant prepared with a 3 mm biopsy punch. Top and side view: the explant cylinder extends from the cartilage surface down close to the subchondral bone. Bar = 1 mm. (b) and (c) Perfusion culture: continuous flow of fresh culture medium through the culture container ensures optimal nutrition of the tissue and swift removal of waste products secreted by the living cells. A peristaltic pump transports culture medium from a storage bottle through the container. Used culture medium is collected in a waste bottle. No reperfusion of culture medium takes place. Cultures are maintained at a constant temperature using a heating plate.

2.4. Paraffin embedding and sectioning

Tissue samples were fixed in 0.4% PFA (Paraformaldehyde) in PBS (phosphate buffered saline) for 12 h at room temperature, then washed in aqua dest. for 12 h. Following dehydration in a graded series of isopropanol ($2 \times 70\%$, $2 \times 80\%$, $2 \times 96\%$, $2 \times 100\%$; 3 h each) the samples were transferred to paraffin via terpenol (3×4 h) as an intermediate. Complete saturation of the tissue with paraffin occurred during a period of 2 days at 60 °C. The samples were then embedded in liquid paraffin at 60 °C in silicone rubber moulds. Hardening of the paraffin occurred at 4 °C for 24 h. For sectioning, the embedded samples were glued onto the sectioning block. Using a HM350 microtome (Microm, Germany), 6-μm-thick sections through the center of the explants were prepared from cartilage surface to bone side, so that rectangular sections were obtained. Sections were then transferred to egg-albumin-treated glass slides and dried at 45 °C for 12 h.

2.5. Histochemical and immunohistochemical analysis

Sections were deparaffinated 2×10 min in 100% xylol and rehydrated in a graded series of ethanol ($2 \times 100\%$, $2 \times 96\%$, $2 \times 80\%$, $2 \times 70\%$; 5 min each). Finally, the sections were transferred to aqua dest. and stained according to histochemical or immunohistochemical protocols. The histochemical staining procedures used were Alcian Blue/van Giesson and Safranin-O to

visualize the amount of matrix glycoproteins within the cartilage sections.

Immunohistochemical analysis was performed using the indirect immunofluorescence method. The deparaffinated and rehydrated sections were incubated in blocking-solution (PBS + 1% BSA + 10% FCS) for 30 min to saturate non-specific binding sites. Primary antibodies were diluted appropriately and applied for 12 h at 4 °C. The primary antibodies used were rabbit–anti-human aggrecan (1:200; generous gift from Dick Heinegård, Institute of Cell and Molecularbiology, University of Lundl, Sweden), mouse–anti-human collagen type II (1:50; ICN Biomedicals Inc., Ohio, USA), goat–anti-human collagen type I (1:20; Chemicon International Inc., CA, USA), rabbit–anti-human COMP (1:50; generous gift from Dick Heinegård, Institut of Cell and Molecularbiology, University of Lund, Sweden) and KI-67 (BD Biosciences/Pharmingen, Germany). Following a washing step in PBS, the fluorochrome-conjugated species-specific secondary antibodies were diluted appropriately and applied for 45 min at room temperature. DAPI (Sigma, Germany) was added in this step at a concentration of 0.5 μg/ml. The secondary antibodies used were TRITC-conjugated goat–anti-rabbit, TRITC-conjugated donkey–anti-goat and FITC-conjugated goat–anti-mouse (All 1:100; Jackson Immunoresearch Laboratories, PA, USA). Following the final washing step, the sections were mounted and analyzed immediately. Specificity of primary antibodies was controlled on fresh cartilage sections, sections from liver, kidney and skin (not shown). Negative controls for all three secondary antibodies were performed by treating sections according to the above protocol, but omitting the primary antibody.

2.6. Evaluation of results

Six sections cut through the center of the explant cylinder from the cartilage surface to the bone side were prepared for analysis with each method. All samples were processed in parallel throughout the staining procedure. Results were evaluated by densitometric analysis using AnalySIS Morphometry Software (Soft Imaging System, Germany) on digital photomicrographs of all stained sections. Camera gain was set to a fixed value for all photographic recordings. Average staining intensity within the extracellular matrix was determined for each section while cellular areas were excluded from analysis by masking (Fig. 2). The entire area of each section was evaluated from surface to bone side and readings were pooled for each section. All three RGB channels were used for analysis of fluorescence micrographs. For analysis of Alcian Blue/van Giesson staining only B-channel (blue) was used while for analysis of Safranin-O staining only R-channel (red) was used. No other image processing was performed. The proportion of staining intensity in each section to staining intensity in the corresponding fresh cartilage control was determined. For further evaluation the mean proportion ($n = 6$) and the range of values was calculated for each experiment. The mitotic index was determined statistically by image analysis from the ratio of KI-67 positive nuclei to DAPI labeled nuclei in each experiment. Using this method the mitotic index was measured in all cultured samples, in freshly isolated articular cartilage and in isolated chondrocytes proliferating in a culture dish in human serum-supplemented medium.

In addition, all stained sections were evaluated according to a grading system by a group of seven unbiased/non-associated researchers and orthopedic surgeons from Gothenburg University, Gothenburg University Hospital, Gothenburg Medical Center and Kungsbacka Hospital (Gothenburg and Kungsbacka, Sweden) in a blind-test where sample names were replaced by codes. Grading was performed on a scale of 5–0, where 5 represented optimal healthy articular cartilage and 0 stood for totally degenerated tissue. For each experiment results from histochemical staining with Alcian Blue and Safranin-O, polarized light

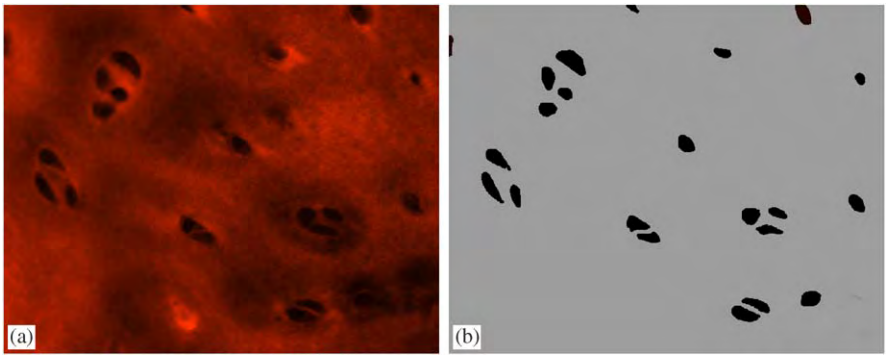

Fig. 2. Densiometric analysis of hyaline cartilage sections stained with antibodies against extracellular matrix components. (a) Indirect immunofluorescence-stained section of human articular cartilage. (b) Mask generated to exclude cellular areas. Only extracellular areas (gray) are used for densiometric analysis.

microscopy, immunohistochemistry against aggrecan, collagen type I, collagen type II and COMP of all samples ($n = 6$) were graded according to this system. A mean value of all grades was calculated for each experiment. The resulting grade value ranged from 5 to 0 (5 = as good as fresh cartilage and 0 = completely degenerated tissue).

3. Results

Cylindrical explants (average diameter: 3 mm; average length 3 mm; average weight: 90 mg) of healthy, intact cartilage (Fig. 1a) were cultured in parallel under different culture conditions for a period of 14, 28, 42 and 56 days, see, Figs. 3 and 4. Static culture experiments were performed in conventional tissue culture dishes using serum-free medium, 10% FCS-supplemented medium or 10% HS-supplemented medium. In the static culture system, the culture medium was renewed every 3–4 days. Perfusion culture experiments were performed in a special bioreactor using serum-free medium (Fig. 1b and c) where the medium was renewed continuously by means of a peristaltic pump. At the end of the culture period, all samples were sectioned and analyzed histochemically and immunohistochemically together with the fresh controls from all

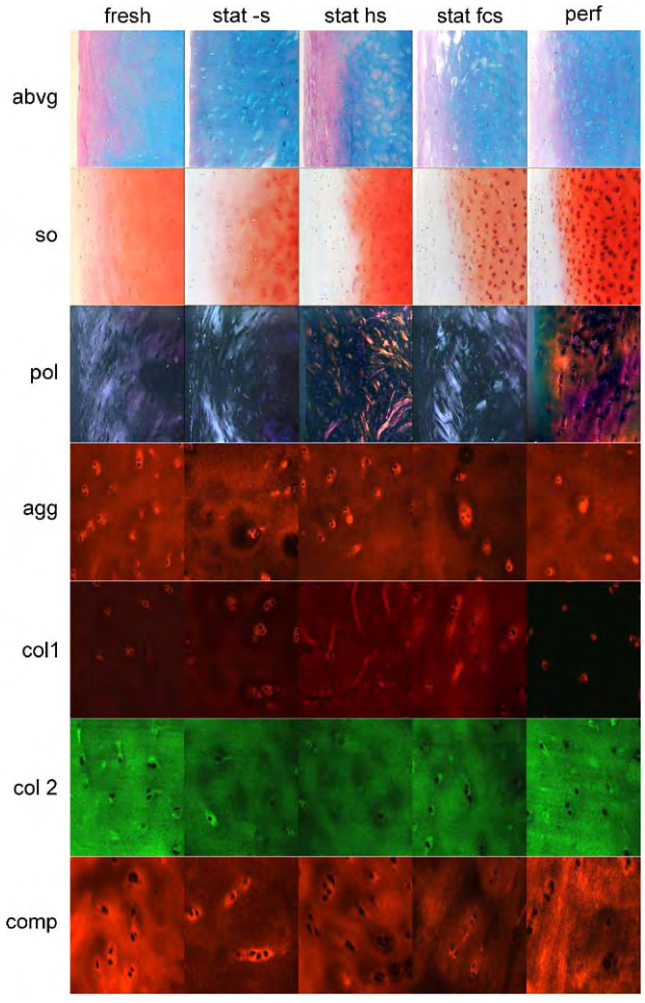

Fig. 3. Exemplary results from fresh hyaline cartilage and after 14 days of culture: Histochemistry with Alcian Blue/van Giesson (abvg) and Safranin-O (so), polarized light microscopy (pol), immunohistochemistry for aggrecan (agg), collagen type I (col 1), collagen type II (col 2) and COMP (comp) shown for static cultures (stat) in DMEM/F12 + 50 µg/ml ascorbic acid + 50 µg/ml gentamycin + 2 µg/ml fungizone serum-free (-s), or + 10% human autologous serum (hs) or + 10% fetal calf serum (fcs) as well as perfusion culture (perf) in serum-free DMEM/F12 + 50 mg/ml ascorbic acid + 1% gentamycin/fungizone. All sections through the center of the explants, cartilage surface (left) to bone side (right).

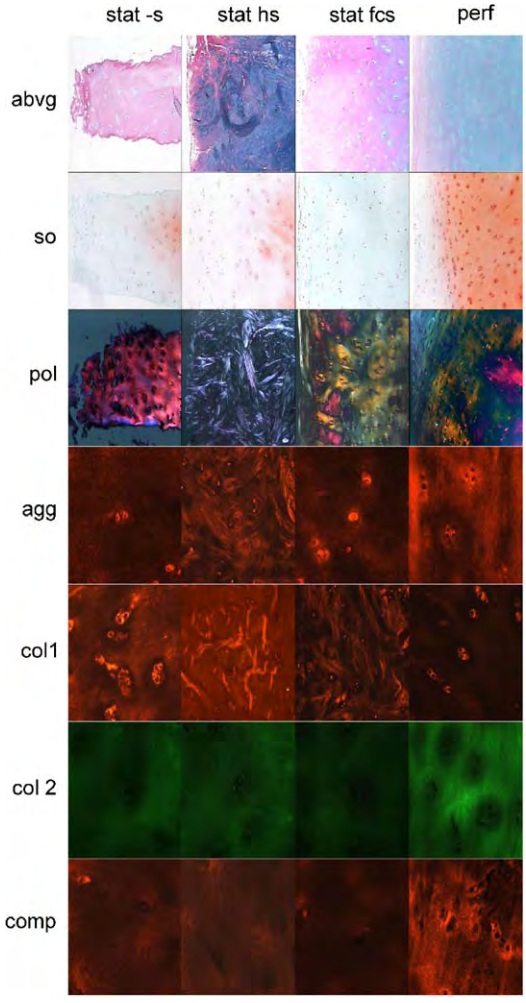

Fig. 4. Exemplary results from hyaline cartilage after 56 days of culture: Histochemistry with Alcian Blue/van Giesson (abvg) and Safranin-O (so), polarized light microscopy (pol), immunohistochemistry for aggrecan (agg), collagen type I (col 1), collagen type II (col 2) and COMP (comp) shown for static cultures (stat) in DMEM/F12 + 50 µg/ml ascorbic acid + 50 µg/ml gentamycin + 2 µg/ml fungizone serum-free (-s), or with 10% human autologous serum (hs) or with 10% fetal calf serum (fcs) as well as perfusion culture (perf) in serum free DMEM/F12 + 50 µg/ml l ascorbic acid + 50 µg/ml gentamycin + 2 µg/ml fungizone. All sections through the center of the explants, cartilage surface (left) to bone side (right).

patients. Evaluation of results was performed by densitometric analysis (Fig. 5), as well as by a grading system (Fig. 6).

3.1. Culture period of 14 days

After 14 days of culture, all specimens were morphologically intact and differences resulting from different culture conditions were small (Figs. 3, 5, and 6). In general, a moderate loss of aggrecan, collagen type II and COMP was apparent under all static culture conditions. Average size and weight of the explants was unchanged compared to fresh controls. Static cultures with 10% human autologous serum showed incipient formation of atypical fibrous structures in polarized light microscopy. This observation was confirmed by an upregulation of collagen type I within fibers as shown by immunohistochemistry (Figs. 3, 5, and 6). Results from all perfusion-cultured specimens on the other hand were difficult to distinguish from the corresponding fresh cartilage controls. KI-67 staining showed an increased mitotic activity in all tissue explants cultured in serum-supplemented media (Table 1). Mitotic activity was highest in human serum-supplemented medium. Samples from serum-free static cultures showed slightly elevated mitosis. Specimens from perfusion culture displayed low mitotic activity comparable to fresh cartilage samples.

3.2. Culture period of 28 and 42 days

Cultures evaluated after 28 and 42 days of culture displayed a clear trend towards the results obtained after 56 days of culture (data from 28 days and 42 days is not shown). Specimens from static cultures showed a continuous loss of typical histochemical matrix staining

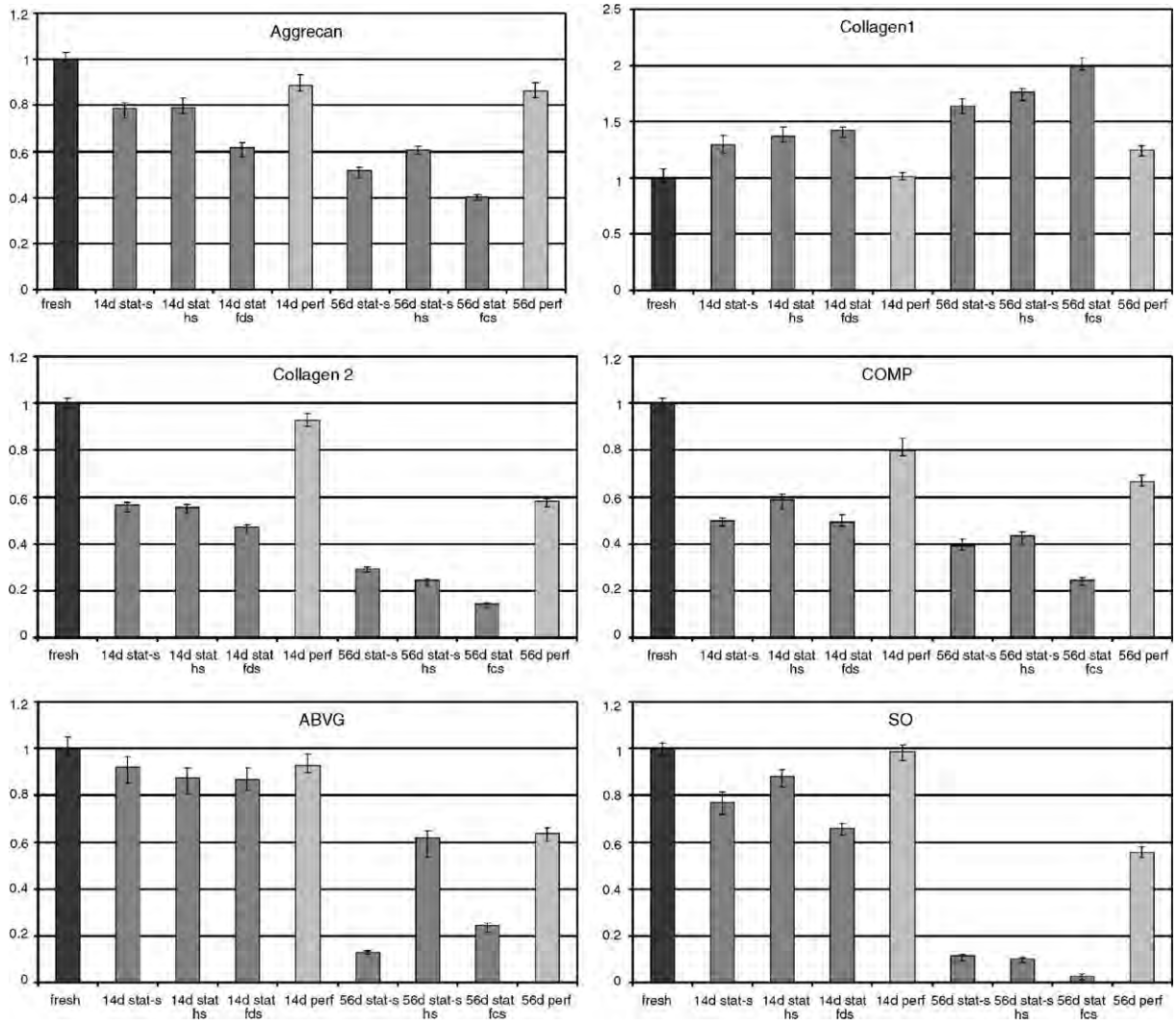

Fig. 5. Graphs summarizing the densiometric analysis of results from immunohistochemistry on hyaline cartilage for aggrecan, collagen type I, collagen type II and COMP as well as histochemistry with Alcian Blue/van Giesson and Safranin-O for each experiment. Bars show the mean ratio of average staining intensity in the extracellular matrix of each experiment to fresh cartilage ($n = 6$). Range of values is shown by range bars.

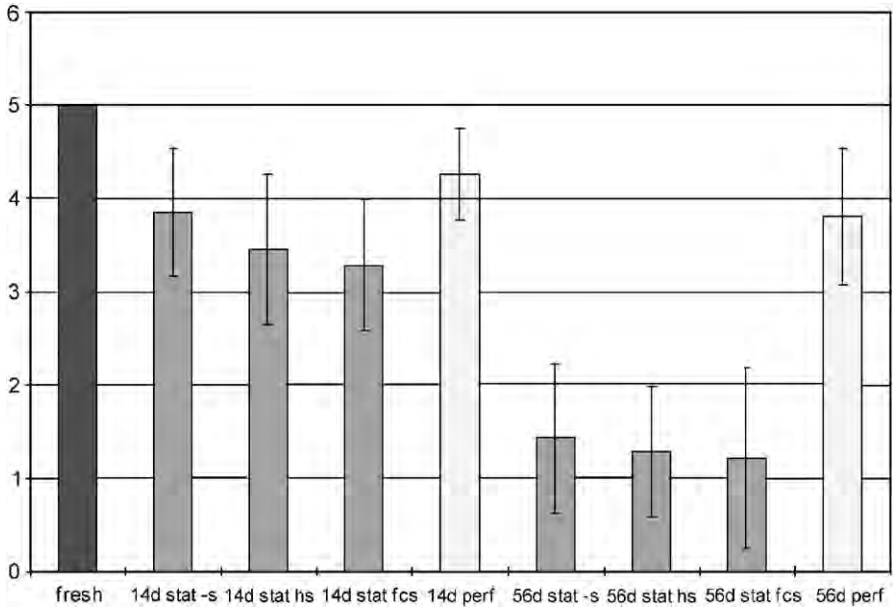

Fig. 6. Graph summarizing the outcome of a blind-test evaluation performed by a group of seven unbiased/non-associated researchers and surgeons for fresh articular cartilage, 14 and 56 d culture time. Grade values range from 5 to 0, where 5 stands for "as good as fresh cartilage" and 0 stands for "completely degenerated". Bars show the average value for each experiment calculated from grades for histochemistry with Alcian Blue/van Giesson and Safranin-O, polarized light microscopy, immunohistochemistry for aggrecan, collagen type I, collagen type II and COMP for six individual samples. Range of values is shown by range bars.

Table 1
Mitotic index of cultured hyaline cartilage as determined from the ratio of KI-67 positive nuclei to DAPI labeled nuclei ($n = 6$)

Fresh	0.06 (±0.09)			
Monolayer	6.03 (±0.36)			
	Static	Static + FCS	Static + HS	Perfusion
14 d	0.58 (±0.14)	2.33 (±0.26)	2.67 (±0.15)	0.22 (±0.09)
28 d	0.61 (±0.17)	1.61 (±0.33)	2.08 (±0.49)	0.28 (±0.17)
48 d	0.39 (±0.20)	1.08 (±0.38)	1.97 (±0.53)	0.08 (±0.09)
56 d	0.33 (±0.21)	1.36 (±0.32)	1.61 (±0.29)	0.14 (±0.07)

The mitotic index of fresh, healthy cartilage explants and the mitotic index of isolated chondrocytes grown as a monolayer in human serum-supplemented medium are included for comparison. Values for the average and standard deviation are given.

as well as a loss of immunohistochemical staining for aggrecan, collagen type II and COMP. Tissue explants cultured in serum-free medium under static conditions started to disintegrate from the periphery after 28 days. Increased formation of atypical fibrous structures along with an increase in collagen type I expression and elevated mitotic activity could be observed in all serum-supplemented static cultures. Specimens cultured under continuous perfusion of serum-free medium in contrast continued to maintain low mitotic activity and hyaline cartilage typical features were preserved.

3.3. Culture period of 56 days

After 56 days of culture the different culture methods had lead to striking differences in the quality of the cultured cartilage tissue (Figs. 4–6). All samples cultured under static conditions showed a definite loss of hyaline cartilage-specific histochemical matrix staining, a clear loss of aggrecan, collagen type II and COMP as well as an upregulation of hyaline cartilage atypical collagen type I (Figs. 4–6). Size and weight of the explants was slightly decreased compared to fresh controls. Serum-free culture under static conditions led to severe disintegration of the explants from the periphery and an extensive decrease in tissue mass. The size of the explants was decreased to an average of 2.5 mm length and 1.7 mm diameter. The weight had decreased to an average of 44 mg.

In polarized light microscopy, samples from static cultures in the presence of 10% HS displayed a great extent of elongated fibrous structures resembling collagen type I fibers in fibrocartilage. This finding was confirmed by a strong expression of fiber-associated collagen type I detected in immuno-histochemistry (Figs. 4–6). Similar results were obtained from

specimens cultured under static cultures in the presence of 10% FCS which also displayed a grainy and inhomogeneous matrix in polarized light microscopy along with fiber-associated collagen type I in immunohistochemistry (Figs. 4–6). Mitotic activity was still clearly elevated in all samples from serum-supplemented static cultures compared to the samples from serum-free cultures and to fresh articular cartilage (Table 1).

Specimens cultured under continuous perfusion in serum-free medium yielded the best results. Average size and weight of the explants was unchanged compared to fresh controls. The samples showed only moderate loss of hyaline cartilage-specific histochemical staining as well as good maintenance of aggrecan, collagen type II and COMP expression within the matrix (Figs. 4–6). Collagen type I upregulation was not detectable under perfusion culture conditions, the matrix appeared homogeneous in polarized light microscopy and Ki-67 immunohistochemistry did not reveal elevated mitotic activity (Table 1). In general, all hyaline cartilage typical features were well preserved and results were most comparable to the corresponding fresh cartilage controls.

4. Discussion

Our goal is to develop a cartilage culture system that can be used to use functional cartilage for testing in vitro over extended periods of time. This culture system should make it possible to test chronic exposure of cartilage to biomaterials. Such a system could be used to study biomaterial–cartilage interaction, biomaterial integration as well as toxicity. One of the most important prerequisites of such a system is the ability to maintain cartilage in vitro in a functionally differentiated state over a period of months in order to generate results that are transferable to the in-vivo situation. Our experiments showed that short-term maintenance of human articular cartilage in a differentiated state is possible under all culture conditions tested. For up to 14 days, morphology as well as degree of differentiation could be maintained at a relatively high level independent of the culture method (Figs. 3, 5 and 6).

For long-term culture up to 56 days on the other hand the perfusion culture clearly yielded the best results. Morphological and histological appearance of the tissue was excellent and cartilage-specific immunohistochemical staining was most comparable to fresh explants. The loss of specific hyaline cartilage features was slow and synthesis of atypical collagen type I was suppressed (Figs. 4–6).

4.1. Mitotic activity influences degree of differentiation

The outcome from conventional static cultures with and without serum proved completely unacceptable for a long-term in vitro experimental procedure. Cartilage cultured under static conditions showed massive dedifferentiation when cultured in serum-supplemented medium whereas it went through morphological disintegration in serum-free medium. Features characteristic for hyaline cartilage were almost lost completely (Figs. 4–6). Aggrecan, collagen type II, and COMP rapidly disappeared from the matrix. At the same time hyaline cartilage atypical collagen type I fibers started to appear.

For the in vitro expansion of undifferentiated cells where high growth rates are desirable, culture media are usually supplemented with serum such as FCS or HS. Sera are rich in growth factors and are highly effective in stimulating cell proliferation [23]. For the culture of differentiated cartilage, on the contrary, permanent mitotic stress has an adverse effect because cells within adult tissues are normally arrested in the G_0-phase [24]. In healthy hyaline cartilage, in particular, no significant cell proliferation can be detected in vivo. As shown by KI-67 labeling, the mitotic index is close to zero (Table 1).

When chondrocytes within intact hyaline cartilage are exposed to the various growth factors present in serum, the cells are driven from the G_0-phase into mitosis [25–27]. A clear increase of mitotic activity could be seen in such explants (Table 1). As the cells start to proliferate they lose their high degree of differentiation and are thus unable to keep up sufficient tissue-specific functions. Even though adult hyaline cartilage matrix has a slow turnover in vivo, the key to maintaining normal hyaline matrix composition is the specific activity of the differentiated chondrocytes embedded within. These postmitotic cells regulate the continuous degradation by matrix metalloproteinases and other enzymes [28,29] as well as the renewal of the surrounding matrix by biosynthesis [30]. If these cells are unable to maintain normal function, e.g. due to a strong mitogenic drive, the matrix composition will start to change over time.

4.2. Dedifferentiation leads to matrix degeneration

The correlation between the dedifferentiation of the chondrocytes and the degeneration of the matrix was apparent in our experiments. The mitotic index in cartilage explants cultured in serum-supplemented medium under static conditions was already high after 14 days (Table 1), whereas serious loss of hyaline cartilage-specific matrix components became visible after 28 to 56 days (Figs. 3–6). Interestingly these changes closely resemble those taking place in degenerative diseases of hyaline cartilage [31,32]. In order to maintain a low mitogenic drive and thus a high degree of differentiation of the tissue in vitro over time, the culture medium should not be supplemented with serum or mitogenic growth factors. The experiments also showed

though, that long-term serum-free culture of cartilage explants is not possible under static conditions. In contrast, specimens cultured in serum-free medium under static conditions did not display elevated mitotic activity and the accompanying changes in matrix composition observed in serum-supplemented cultures. However, such culture conditions on the other hand caused significant tissue disintegration as well as cell death along with a marked reduction in tissue mass.

Constant supply of nutrition, swift removal of harmful metabolites and the effective prevention of an accumulation of paracrine factors are known important requirements in tissue culture [33]. These issues are addressed by the use of perfusion culture that maintains a continuous flow of fresh medium [34]. As demonstrated by the long-term results obtained from perfusion cultured explants, dynamic fluid exchange apparently improves the culture environment as well as diffusion into and out of the tissue (Figs. 4–6). Thus, necrosis and apoptosis due to malnutrition and self-toxication can be prevented even in 3 mm diameter explants. The chondrocytes maintain a differentiated phenotype demonstrated by the maintenance of a hyaline cartilage-specific extracellular matrix.

Perfusion culture under serum-free conditions forms a promising basis for a long-term in vitro model system to test the biocompatibility of scaffolds or membranes for application inside the joint. As cartilage is a slowly reacting tissue, a long-term model system is necessary to perform in vitro tests over extended periods of time in order to collect realistic biocompatibility data. In vitro test cultures in which for example an intact cartilage explant is cultured in direct contact with a biomaterial could be performed for several months instead of weeks. At the same time, such a long-term culture system could be used to investigate the slowly acting mechanisms of cartilage degeneration and repair under defined conditions. Applications of this long-term culture system in cartilage tissue engineering remain to be evaluated. It is known that integration of in vitro-engineered cartilage into cartilage in vivo actually becomes problematic when the engineered construct is too mature [35,36]. Optimally the duration of the in vitro perfusion culture should be adjusted in these applications to obtain half-mature constructs for transplantation.

In general, the results show that reduced mitogenic drive (= serum-free conditions) in combination with continuous medium renewal, drastically improves the quality of the cultured hyaline cartilage in long-term experiments. In addition to the culture parameters considered in this set of experiments, cyclic mechanical loading [37,38], controlled oxygen tension [39,40], as well as optimized temperature, pH and medium composition [41,42] to better resemble the situation within the joint can further improve the culture environment and make the model even more realistic. In a future biocompatibility test, the morphological, biochemical and immunohistochemical analysis can be complemented with an analysis of gene expression.

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