CARTILAGE TISSUE ENGINEERING USING PRE-AGGREGATED HUMAN ARTICULAR CHONDROCYTES

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Abstract

In this study, we first aimed at determining whether human articular chondrocytes (HAC) proliferate in aggregates in the presence of strong chondrocyte mitogens. We then investigated if the aggregated cells have an enhanced chondrogenic capacity as compared to cells cultured in monolayer. HAC from four donors were cultured in tissue culture dishes either untreated or coated with 1% agarose in the presence of TGFβ-1, FGF-2 and PDGF-BB. Proliferation and stage of differentiation were assessed by measuring respectively DNA contents and type II collagen mRNA. Expanded cells were induced to differentiate in pellets or in Hyaff®-11 meshes and the formed tissues were analysed biochemically for glycosaminoglycans (GAG) and DNA, and histologically by Safranin O staining. The amount of DNA in aggregate cultures increased significantly from day 2 to day 6 (by 3.2-fold), but did not further increase with additional culture time. Expression of type II collagen mRNA was about two orders of magnitude higher in aggregated HAC as compared to monolayer expanded cells. Pellets generated by aggregated HAC were generally more intensely stained for GAG than those generated by monolayer-expanded cells. Scaffolds seeded with aggregates accumulated more GAG (1.3-fold) than scaffolds seeded with monolayer expanded HAC. In conclusion, this study showed that HAC culture in aggregates does not support a relevant degree of expansion. However, aggregation of expanded HAC prior to loading into a porous scaffold enhances the quality of the resulting tissues and could thus be introduced as an intermediate culture phase in the manufacture of engineered cartilage grafts.

Keywords: articular chondrocytes, chondrogenesis, cell differentiation, cartilage tissue engineering.

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Introduction

Cartilage repair techniques based on the use of autologous chondrocytes, either directly injected into the defect or further processed to engineer cartilaginous grafts, typically rely on the *ex vivo* expansion of the limited number of cells that can be obtained from a small biopsy. However, chondrocyte expansion is intrinsically associated with the well-known problem of cell de-differentiation (Benya and Shaffer, 1982; Binette *et al*., 1998), and the re-differentiation capacity of expanded cells, especially if of human origin, is rather limited (Bonaventure *et al*., 1994). In order to enhance the chondrogenic ability of expanded chondrocytes, several strategies have been proposed with various degrees of success, including medium supplementation with specific growth factors (Jakob *et al*., 2001; Barbero *et al*., 2003; Malpeli *et al*., 2004), cell growth on microcarrier beads (Malda *et al*., 2003) or on collagen type II-coated substrates (Barbero *et al*., 2006).

Alternatively, considering that chondrocyte de-differentiation is linked to the cytoskeletal changes induced by cell spreading on a culture substrate (Mayne *et al*., 1976), chondrocytes could be expanded under conditions inhibiting cell adhesion. The proof of principle of this approach was provided by chondrocyte expansion in alginate beads (Lee *et al*., 2003); however, repeated passaging in alginate was necessary to obtain relevant numbers of cells. A more simple implementation of the same principle would be offered by chondrocyte culture on agarose coated dishes. Using this culture model, human articular chondrocytes (HAC) have been shown to form three-dimensional (3D) aggregates, also referred to as clusters or spheroids, and to maintain the expression of cartilage specific proteins (Kolettas *et al*., 1995; Anderer and Libera, 2002). However, little or no proliferation was reported (Kolettas *et al*., 1995).

In this study, we first aimed at determining whether in the presence of a combination of strong chondrocyte mitogens it is possible to achieve HAC proliferation in aggregate cultures. We then investigated whether the aggregated cells have an enhanced chondrogenic capacity as compared to cells cultured using traditional monolayer techniques. A *partial* expansion in monolayer prior to the culture phase in aggregate was introduced to permit more extensive cell proliferation while minimizing chondrocyte de-differentiation (Fig. 1). The chondrogenic ability of the expanded cells was then tested in a standard pellet culture model or using a porous 3D scaffold, that would permit the generation of relatively large cartilage grafts with a limited amount of chondrocyte aggregates.

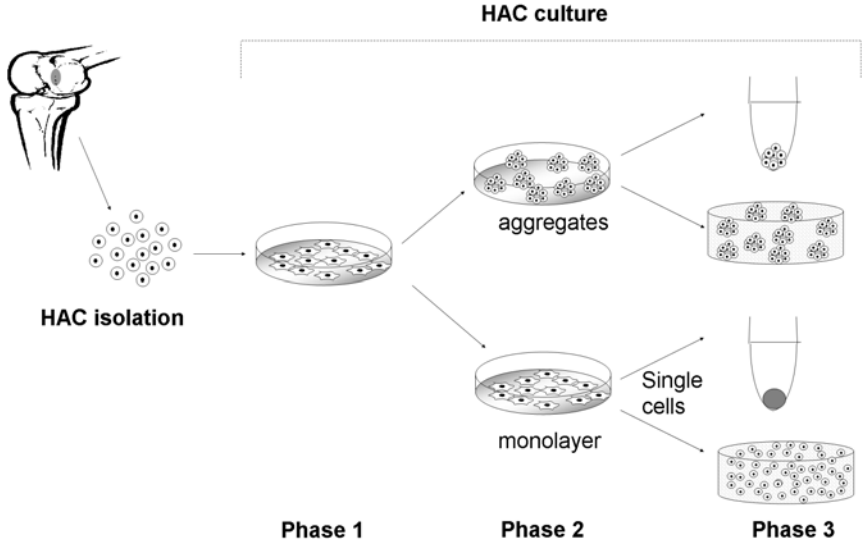

Figure 1. Experimental design. Human articular chondrocytes (HAC) isolated from cartilage biopsies (N = 4) were expanded for one passage in monolayer (*Phase 1*) and then cultured in dishes either uncoated or coated with agarose to generate monolayer cells or form aggregates, respectively (*Phase 2*). At the end of *Phase 2*, aggregates or monolayer expanded chondrocytes were induced to redifferentiate in a pellet culture model or within Hyaff®-11 non-woven meshes (*Phase 3*).

Materials and Methods

Cartilage collection and cell isolation

Human articular cartilage samples (approximately 200 mg tissue) were collected after informed consent and in accordance with the local Ethical Commission from the knee joints of 4 individuals with no known clinical history of joint disorders (*donor 1*: female, 51 years; *donor 2*: female, 67 years; *donor 3*: male, 71 years, *donor 4*: male, 18 years). Chondrocytes were isolated using 0.15% type II collagenase (Bioconcept Worthington, Allschwill, Switzerland) for 22 hours and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing, 4.5 mg/ml D-glucose, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.29 mg/ml L-glutamine (complete medium) and 10% foetal bovine serum (FBS).

Cell expansion and aggregation

Human articular chondrocytes (HAC) were plated on tissue culture dishes at 10^4 cells/cm^2 in 10% FBS supplemented complete medium with the addition of 5 ng/ml of Fibroblast Growth Factor-2, 10 ng/ml of Platelet-Derived Growth Factor-bb and 1 ng/ml of Transforming Growth Factor-β1 (all from R&D Systems, Minneapolis, MN, USA), previously reported to be potent mitogens for HAC (Jakob *et al.*, 2001), and cultured until they reached about 80% confluence (7-10 days; *Phase 1*, see Fig.1). HAC were then detached using 0.3% type II collagenase and 0.05% trypsin/0.53mM EDTA (Gibco; Invitrogen, Basel, Switzerland) and replated at $5x10^3$ cells/cm^2 in culture dishes either untreated or coated with 1.0% agarose (Sigma-Aldrich, Hamburg, Germany), in order to support respectively cell growth in monolayers or the formation of aggregates (up to 14 days; *Phase 2*, see Fig.1). Culture medium during *Phase 2* was also supplemented with the 3 growth factors used for *Phase 1*. At each feeding, aggregates collected in the spent medium were resuspended in fresh medium and returned to the culture dish. Cells cultured in monolayer or in aggregates were either processed for gene expression analysis or induced to re-differentiate using the two models described below (*Phase 3*, see Fig. 1).

Cell redifferentiation

Pellet culture. For this differentiation assay, cells from 3 donors (*donor 1*, *donor 2* and *donor 3*) were used. Monolayer- or aggregate-cultured cells were transferred into polypropylene conical tubes (respectively $5x10^5$ cells/pellet and 1 to 4 aggregates/pellet, depending on their size) in the presence of complete medium supplemented with insulin, transferrin and selenous acid (ITS^{+1}, Sigma Chemical, St. Louis, MO, USA), 0.1 mM ascorbic acid 2-phosphate, 1.25 mg/ml human serum albumin, 10^{-7} M dexamethasone and 10 ng/ml Transforming Growth Factor-β1 to establish pellet cultures, as previously described (Barbero *et al.*, 2004). Pellets were placed onto a 3D orbital shaker (Bioblock Scientific, Frenkendorf, Switzerland) at 30 rpm and cultured for 2 weeks in 0.5 ml of medium/pellet, with medium changes twice per week. Subsequently pellets were processed for histological or biochemical analysis.

3D culture within scaffolds. For this differentiation assay, cells from 2 donors (*donor 3* and *donor 4*) were used. Monolayer- or aggregate-cultured cells were seeded on Hyaff®-11 non-woven meshes (8 mm diameter, 2 mm thick discs; Fidia Advanced Biopolymers, Abano Terme, Italy) by direct perfusion through the scaffold pores. 4 or 8 millions of HAC as single cells or as aggregates were

perfused in alternate directions through each mesh in 4 ml of culture medium at a superficial velocity of 3 ml/min for 24 hours (Wendt *et al.*, 2003). The number of cells in aggregates was estimated by measuring the DNA contents of aliquots of cell-cluster suspension on the day before the seeding. After perfusion seeding the resulting cell-scaffold constructs were processed histologically to examine the distribution of the cells thought the scaffold, by quantifying the DNA content to assess the cell seeding efficiency, or transferred on agarose coated dishes and cultured in complete medium supplemented with 10% FBS, 0.1 mM ascorbic acid 2-phosphate, 10 mg/ml Insulin and 10 ng/ml Transforming Growth Factor-b3 for 4 weeks with medium changes twice per week (Candrian *et al.*, 2008).

Analyses of cells and tissues

Real-time quantitative RT-PCR assays. Total RNA of cells after *Phase 2* (see Fig. 1) was extracted using Trizol (Life Technologies, Basel, Switzerland) and the standard single-step acid-phenol guanidinium method. RNA was treated with DNAseI using the DNA-free™ Kit (Ambion/ Applied Biosystems, Foster City, CA, USA) and quantified spectrometrically. cDNA was generated from 3 µg of RNA by using 500 µg/ml random hexamers (Catalys AG, Wallisellen, Switzerland) and 1 µl of 50 U/ml Stratascript™ reverse transcriptase (Stratagene, Amsterdam, the Netherlands), in the presence of dNTPs. Real-time RT-PCR reactions were performed and monitored using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer/ Applied Biosystems, Rotkreuz, Switzerland). After an initial denaturation at 95°C for 10 min, the cDNA products were amplified with 45 PCR cycles, consisting of a denaturation step at 95°C for 15 s and an extension step at 60°C for 1 min. The following primers and probes were used at the concentrations indicated in parenthesis:

CII-forward primer:
5'-GGCAATAGCAGGTTCACGTACA (900 nM),
CII-reverse primer:
5'-CGATAACAGTCTTGCCCCACTT (900 nM),
CII-probe:
5'-CCGGTATGTTTCGTGCAGCCATCCT (200 nM);
18-S-forward primer:
5'-CGGCTACCACATCCAAGGAA (26 nM),
18-S-reverse primer:
5'-GCTGGAATTACCGCGGCT (26 nM),
18-S-probe:
5'-TGCTGGCACCAGACTTGCCCTC (50 nM).

For each sample, the threshold cycle (Ct) value was determined as the cycle number at which the fluorescence intensity reached 0.05. For each cDNA sample, the value of 18-S was subtracted from the Ct value of the target gene, to derive µCt. The level of expression of type II collagen was calculated as $2^{\mu Ct}$. Each sample was assessed at least in duplicate.

DNA and Glycosaminoglycan (GAG) quantification. Aggregates generated at different times during *Phase 2* (i.e., at day 0, 2, 4, 6, 8 and 14), pellets cultured for 2 weeks and cell-scaffold constructs immediately after seeding or 4 weeks's culture were digested with protease K (0.5 ml of 1 mg/ml protease K in 50 mM Tris with 1 mM EDTA, 1 mM iodoacetamide, and 10 µg/ml pepstatin-A for 15 hours at 56°C) as previously described (Hollander *et al.*, 1994). The DNA amount was measured spectrofluorometrically using the CyQUANT® Kit (Molecular Probes, Eugene, OR, USA) following the kit's instruction. Briefly, CyQUANT® NF dye reagent was applied to cell lysets aliquoted in a 96-well plate (3 wells per sample). After a 30 minute incubation, fluorescence was measured (excitation: 485 nm; emission: 530 nm) and compared to standard curve prepared simultaneously using calf thymus DNA.

Glycosaminoglycan (GAG) contents of pellets and constructs were measured spectrophotometrically using the dimethylmethylene blue (DMMB) assay (Farndale *et al.*, 1986). Briefly, DMMB solution was applied to cell lysets aliquoted in a 96-well plate (4 wells per sample). Absorbance was measured at 525 nm and compared to curve prepared simultaneously using chondroitin sulphate (Sigma-Aldrich). GAG contents of pellets and cell-scaffold constructs are reported as µg GAG / µg DNA.

Histological analysis. Cell pellets and cell-scaffold constructs were fixed in 4% formalin, embedded in paraffin, cross-sectioned (5 µm thick) and stained with Safranin O for sulphated GAG.

Statistical Analysis. All values are presented as mean ± standard deviation of measurements from 3 independent experiments (i.e., with cells from the 3 different donors). Differences among experimental groups were assessed by Mann Whitney tests, and considered statistically significant with $P < 0.05$.

Results and Discussion

Proliferation and differentiation stage of HAC cultured in monolayer or in aggregates

In the first set of experiments HAC were cultured in monolayer or on agarose coated dish in the presence of a growth factor combination (namely TGFβ-1, FGF-2 and PDGF-BB) known to strongly enhance the proliferation rate of monolayer cultured chondrocytes (Barbero *et al.*, 2003). HAC cultured on agarose coated surfaces remained non-adherent and formed aggregates growing in size with time, in agreement with previous studies (Archer *et al.*, 1990; Izumi *et al.*, 2000; Kolettas *et al.*, 1995; Anderer and Libera, 2002). In particular, during the first 6 days of aggregate culture cells generated rather uniformly sized aggregates (size 15-70 µm), which during the second week of expansion tended to fuse together and form larger aggregates, with highly variable sizes (Fig. 2A). The amount of DNA in aggregate cultures increased significantly from day 2 to day 6 (by 3.2-fold), but did not further increase with additional culture time (Fig. 2B). During the first 6 days of culture in *Phase 2*, as compared to monolayer-expanded cells, the proliferation rate of HAC cultured in aggregates was 5.4-fold lower (Fig. 2C), whereas the expression of collagen type II mRNA was about two orders of magnitude higher (Fig. 2D), and only about 5-fold lower than in native cartilage tissues. Our findings are in general agreement with those by Kolettas *et al.* (1995), reporting that chondrocytes in aggregates

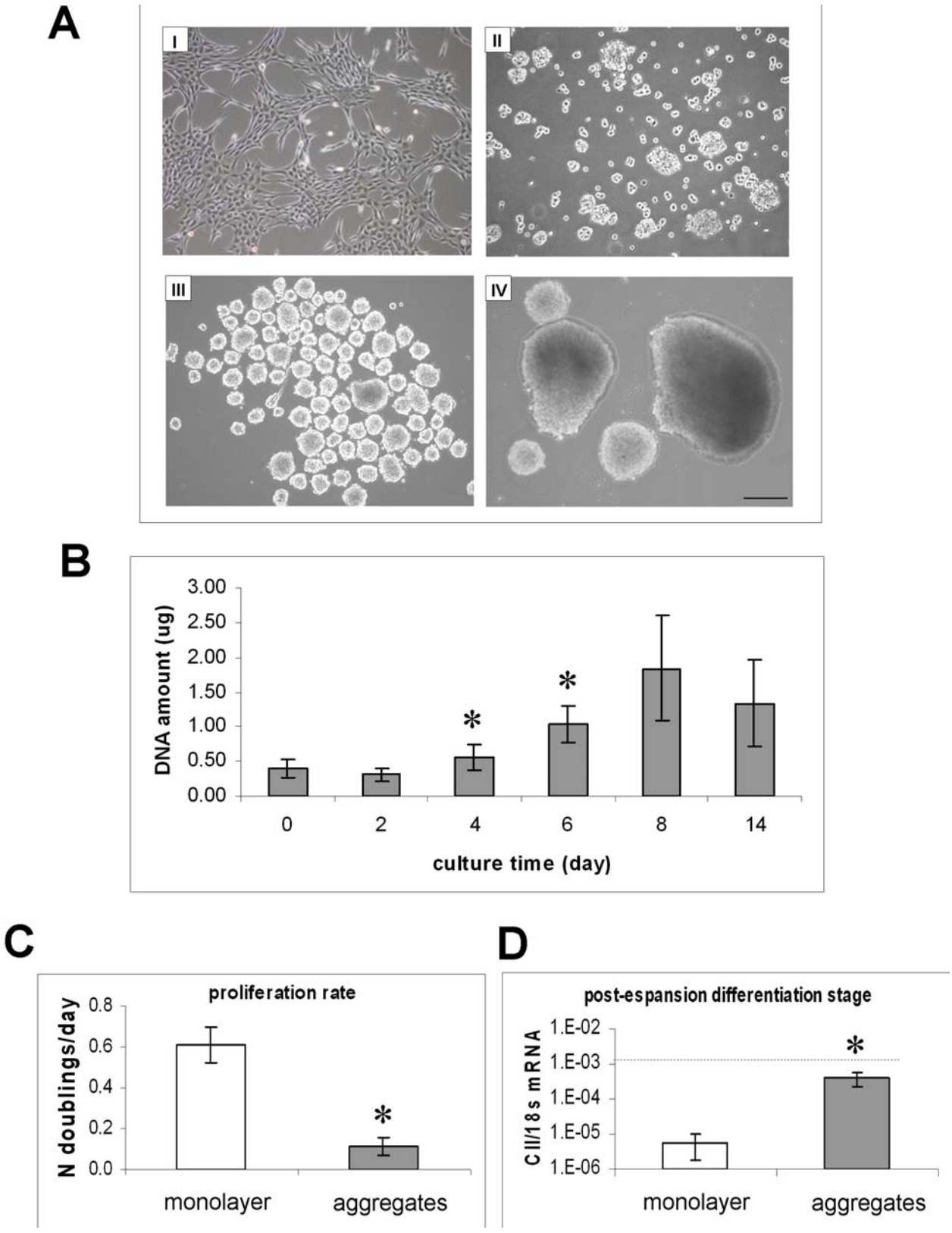

Figure 2. Morphology, proliferation capacity and phenotype of human articular chondrocytes (HAC) expanded in monolayer or aggregates. (**A**) Representative pictures of monolayer (**I**) and aggregates (**II**, **III** and **IV**) formed by HAC at day 3 (**II**), 6 (**I** and **III**), or 14 (**IV**) of culture in *Phase 2*. Bar = 100 µm. (**B**) DNA quantification of HAC cultured as aggregates for different time points. * = $p<0.05$ from the previous time point. (**C**) Proliferation rates of HAC expanded as monolayer or aggregates. * = $p<0.05$ from monolayer. (**D**) Real time RT-PCR analysis of the mRNA expression of collagen type II by HAC after 6 days of culture in *Phase 2*. The dashed line indicates the mean collagen type II mRNA expression level of corresponding native tissues. * = $p<0.05$ from monolayer.

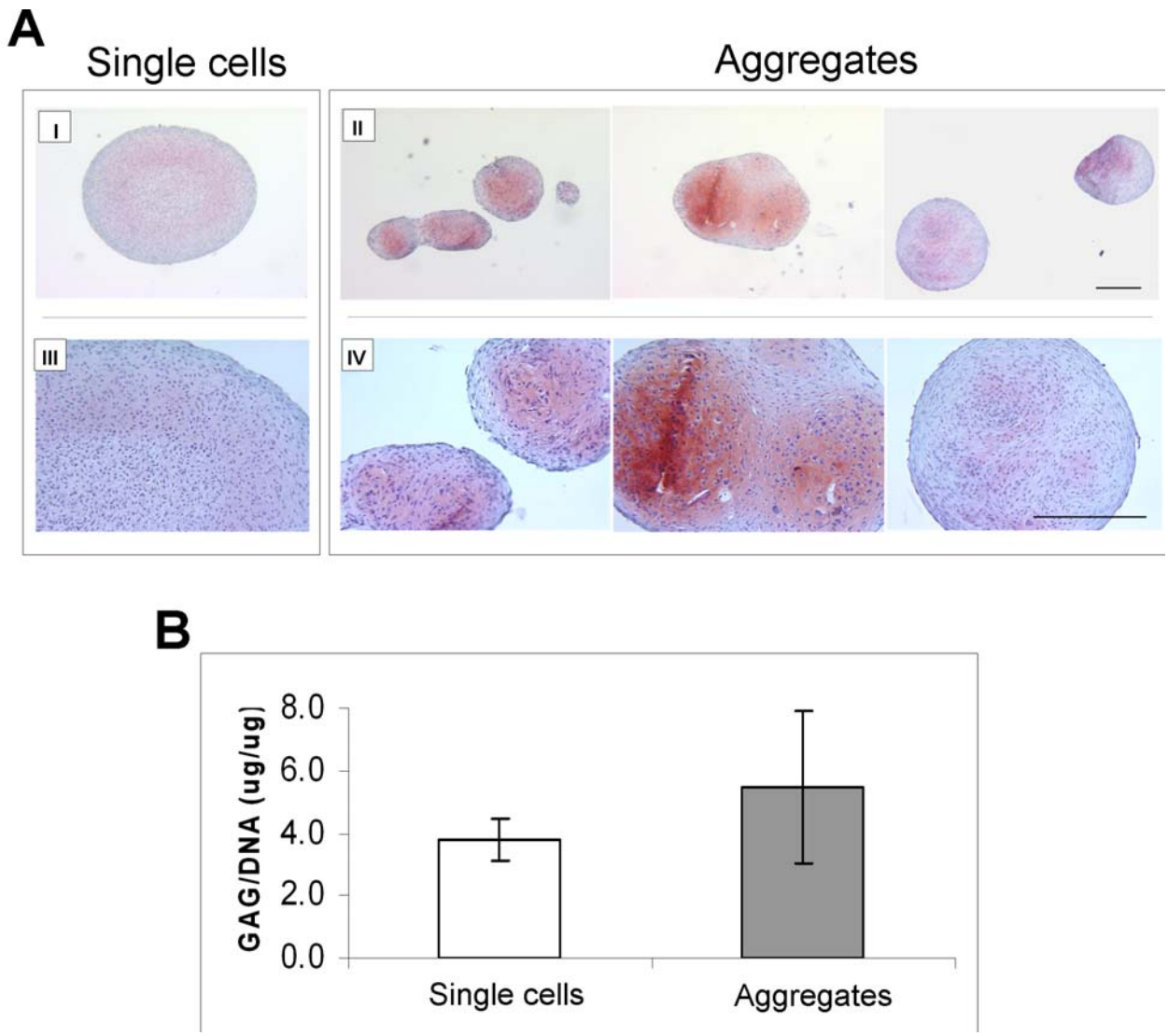

Figure 3. Redifferentiation of human articular chondrocytes (HAC) cultured as monolayer or aggregates in pellets. (**A**) Safranin O staining of representative pellets generated by HAC cultured as monolayer (**I** and **III**) or aggregates (**II** and **IV**) after two weeks' culture in chondrogenic medium. Bars = 100 µm. (**B**) Sulphated glycosaminoglycan (GAG) content, normalized to the DNA content, of the pellets generated by HAC expanded as monolayer or aggregates after two weeks' culture in chondrogenic medium.

expressed higher amounts of collagen type II both at mRNA and protein level than those cultured in monolayer. The limited proliferative activity of chondrocytes in aggregates, despite the medium supplementation with potent mitogens, is also consistent with previous reports on disc cells, which were found to weakly respond to the mitogenic factor PDGF when cultured in alginate (Gruber and Hanley, 2000).

Since aggregate culture did not result in relevant proliferation (i.e., only about 1 doubling in the first 6 days), for subsequent experiments of tissue formation (*Phase 3*) we compared cells cultured as monolayer or as aggregates in *Phase 2* only for 3 days. Thus, for these experiments aggregation in *Phase 2* was not used to further expand cells, but only to pre-condition cells for subsequent chondrogenesis assays.

Redifferentiation of HAC cultured in monolayer or in aggregates

Pellet culture. HAC from three donors cultured in monolayer or aggregates for 3 days in *Phase 2* were transferred into a pellet culture model and induced to redifferentiate in the presence of chondrogenic stimuli. After 2 weeks' culture, pellets generated by aggregated HAC were generally more intensely stained for GAG than those generated by monolayer-expanded cells isolated from the same donors, although variable levels of GAG staining intensity were observed in pellets derived by different aggregates from the same primary culture (Fig 3A). Thus, no statistically significant difference in the biochemically determined GAG/DNA could be established (Fig. 3B). The variations observed among aggregates might be due to the fact that they were formed by distinct sub-populations of chondrocytes present within native cartilage (Barbero *et*

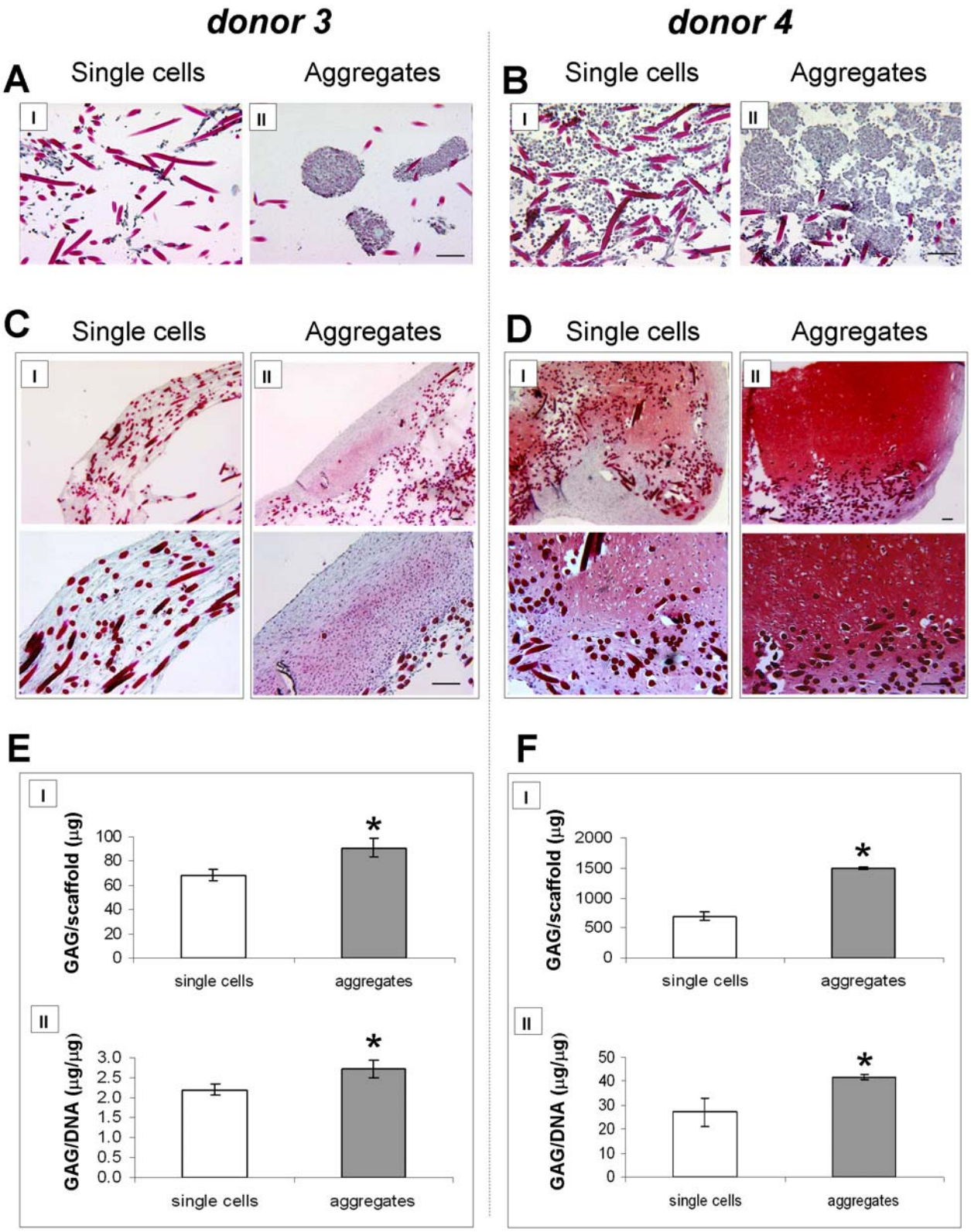

Figure 4. Redifferentiation of human articular chondrocytes (HAC) derived from *donor 3* (71 y) or *donor 4* (18 y) seeded as monolayer or aggregates within a scaffold. (**A-B**) Distribution of HAC thought the Hyaff®-11 non-woven meshes following 24 hours perfusion seeding of cells cultured as monolayer (**I**) or aggregates (**II**). (**C-D**) Safranin O staining of representative constructs generated by HAC cultured as monolayer (**I**) or aggregates (**II**) after two weeks' culture in chondrogenic medium. Bars = 100 µm. (**E-F**) Sulphated glycosaminoglycan (GAG) content of the resulting tissues, reported as total amounts / scaffold (**I**) or after normalization to the DNA content (**II**).

al., 2003; Thornemo *et al.*, 2005), or that they were at different stages of development upon transfer into pellets. Alternatively, since the number of chondrocytes per aggregate could not be controlled, the variability in the chondrogenesis among aggregates might have resulted from differences in their size, in turn affecting patterns of nutrition and/or generation of oxygen gradients.

3D culture within scaffolds. HAC from *donor 3* (71 years) were cultured in monolayer or aggregates for 3 days in *Phase 2* and then seeded within Hyaff®-11 meshes under direct perfusion at a density of 4 millions of cells per scaffold. The efficiency of seeding of both monolayer- or aggregate-cultured chondrocytes, calculated by assessing the DNA content of seeded constructs, was consistently greater than 90%. After 4 weeks' culture in chondrogenic medium, cells expanded in monolayer remained fibroblastic and the resulting tissues were only faintly stained for GAG (Fig. 4C). In contrast, HAC cultured in aggregates for 3 days had a more round morphology and generated scattered areas of Safranin O-positive matrix. Biochemical analyses confirmed a significantly higher accumulation of GAG in scaffolds seeded with aggregate- as compared to monolayer-cultured cells (Fig. 4E). The overall limited extent of chondrogenesis, under similar conditions previously reported to support cartilage tissue formation, can be explained by the rather old age of the donor (Barbero *et al.*, 2004) and/or the low cell seeding density used (Moretti *et al*., 2005) (Fig 4A). We thus performed one additional experiment in which cells derived from a younger donor (*donor 4*, 18 years) were seeded at higher density in the scaffold (i.e.: 8 millions cells/scaffold) as single cells (i.e.: cells cultured in monolayer) or aggregates. Histological analysis of scaffolds after 24 hours perfusion seeding confirmed that scaffolds seeded with HAC from *donor 4* were more cellular than those seeded with HAC from *donor 3* and indicated that, while single cells were uniformly distributed through the scaffolds, aggregates were mainly concentrated at the periphery (Fig 4A-B). Despite the generally higher extent of chondrogenesis by cells from *donor 4* in each culture condition, the trend of improved deposition of GAG following pre-aggregation was confirmed (Fig. 4D and F). Enhancement of chondrogenesis following cell aggregation may be explained by early upregulation of cell-cell and cell-matrix adhesion molecules, which are known to be key to initiate the chondrocyte differentiation program during mesenchymal condensation (Tavella *et al*., 1997). This interpretation would be consistent with a less marked effect of HAC aggregation in the pellet culture model, where the high cell density would already induce upregulation of such molecules (DeLise *et al*., 2000). The results are also consistent with the findings of Masuda *et al.* (2003) that a first step of chondrocyte culture in alginate induces the production of a proteoglycan-rich cell associated matrix (CM) and the formation of cartilaginous structures following culture of the recovered cells and CM from the beads within a porous membrane. Preferential chondrogenesis at the periphery of the aggregate-seeded scaffolds could be explained by an initially higher cell density in the outer region of the constructs, which in turn could result from a limited penetration of the aggregates through the scaffold pores during seeding, and/or from limited nutrition of cells in the interior regions. A higher uniformity of the engineered cartilage grafts could thus be obtained by (i) standardizing the size and shape of the aggregates into a suitable range (e.g., by using hanging drop methods (Kelm *et al*., 2003; Del Duca *et al.*, 2004)), (ii) using scaffolds with a more open pore structure (e.g., rapid-prototyped structures with grid shapes (Woodfield *et al*., 2004)), and/or (iii) increasing mass transport through the constructs (e.g., continuing the perfusion regime following cell seeding (Wendt *et al*., 2006)).

Conclusions

We have shown that even in the presence of strong chondrocyte mitogens, HAC in aggregate cultures undergo a limited extent of growth, but their dedifferentiation is strongly reduced as compared to cells expanded in monolayer for the same time. Aggregation of HAC prior to perfusion-seeding into porous scaffolds enhanced chondrogenesis and allowed generation of cartilage tissues with increased GAG contents. Our study provides a proof-of-principle that HAC expansion in monolayer combined with a subsequent short culture phase in aggregate prior to seeding into a scaffold could be a valid strategy to engineer cartilage grafts starting from the limited number of cells that can be isolated from a small biopsy.

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