Journal of Cellular Biochemistry 81:368–377 (2001)

# Specific Growth Factors During the Expansion and Redifferentiation of Adult Human Articular Chondrocytes Enhance Chondrogenesis and Cartilaginous Tissue Formation in Vitro

**M. Jakob, O. Démarteau, D. Schäfer, B. Hintermann, W. Dick, M. Heberer, and I. Martin***

Department of Surgery, Research Division, University of Basel, Switzerland

**Abstract** Adult human articular chondrocytes were expanded in a medium with 10% serum (CTR) or further supplemented with different mitogens (i.e., EGF, PDGFbb, FGF-2, TGFβ1, or FGF-2/TGFβ1). Cells were then induced to redifferentiate in 3D pellets using serum-supplemented medium (SSM), serum-free medium (SFM), or SFM supplemented with factors inducing differentiation of chondroprogenitor cells (i.e., TGFβ1 and/or dexamethasone). All factors tested during expansion enhanced chondrocyte proliferation and dedifferentiation, as assessed by the mRNA ratios of collagen type II to type I (CII/CI) and aggrecan to versican (Agg/Ver), using real-time PCR. FGF-2/TGFβ1-expanded chondrocytes displayed the lowest doubling times, CII/CI and Agg/Ver ratios, averaging, respectively, 50, 0.2 and 15% of CTR-expanded cells. Redifferentiation in pellets was more efficient in SFM than SSM only for EGF-, PDGFbb- or FGF-2-expanded chondrocytes. Upon supplementation of SFM with TGFβ and dexamethasone (SFM TD), CII/CI ratios decreased 4.4-fold for EGF- and PDGFbb-expanded chondrocytes, but increased 96-fold for FGF-2/TGFβ1-expanded cells. Chondrocytes expanded with FGF-2/TGFβ1 and redifferentiated in SFM TD expressed the largest mRNA amounts of CII and aggrecan and generated cartilaginous tissues with the highest accumulation of glycosaminoglycans and collagen type II. Our results provide evidence that growth factors during chondrocyte expansion not only influence cell proliferation and differentiation, but also the cell potential to redifferentiate and respond to regulatory molecules upon transfer into a 3D environment. J. Cell. Biochem. 81:368–377, 2001. 

**Key words:** serum free medium; cartilage tissue engineering; real time PCR; chondrocyte differentiation, 3D cultures

In the past few years, several studies have underlined the potential of ex vivo engineered cartilaginous grafts to promote a functional repair of large articular cartilage defects [Freed et al., 1994; Kawamura et al., 1998; Sittinger et al., 1999]. In order to generate autologous cartilaginous constructs, however, the limited number of human chondrocytes which are available from a small articular cartilage biopsy needs to be reproducibly and efficiently utilized. Articular chondrocytes, mainly characterized by a spherical cell shape and the synthesis of specific structural macromolecules (i.e., collagen type II and aggrecan), dedifferentiate during in vitro expansion in monolayer culture, assuming a fibroblast-like morphology and expressing macromolecules typical of pre-chondrogenic mesenchymal cells (e.g., collagen type I and versican) [Benya and Shaffer, 1982; Binette et al., 1998]. In principle, chondrocyte dedifferentiation can be reversed by transferring cells into an environment supporting a spherical morphology such as in pellets [Stewart et al., 2000], polymer gels [Benya and Shaffer, 1982], or three-dimensional (3D) scaffolds [Martin et al., 1999; Stewart et al., 2000]. However, the generation of autologous cartilaginous grafts requires the development of procedures not only to quickly expand human chondrocytes, but also to maintain their chondrogenic potential and to reproducibly promote their redifferentiation in a 3D culture system.

It has long been known that human articular chondrocytes can undergo only a limited num-

*Correspondence to: Ivan Martin. Research Division of the Department of Surgery, University of Basel, Hebelstrasse 20, ZLF, Room 405, 4031 Basel, Switzerland.
E-mail: imartin@uhbs.ch

Received 7 June 2000; Accepted 29 September 2000


This article published online in Wiley InterScience, January XX, 2001.

ber of cell divisions in vitro and that their proliferative potential decreases with age [Evans and Georgescu, 1983]. Specific growth factors added to monolayer cultures were reported to induce proliferation of rabbit [Vivien et al., 1990; Bradham and Horton, 1998], bovine [de Haart et al., 1999; Martin et al., 1999] and human [Guerne et al., 1995] articular cartilage cells. These results outlined a possible solution to reproducibly reduce the times of expansion for human chondrocytes, even when isolated from elderly individuals. However, the differentiation stage and redifferentiation potential of human articular chondrocytes expanded in the presence of different growth factors have not yet been investigated.

The redifferentiation of human expanded chondrocytes in vitro was reported to be negligible [de Haart et al., 1999], to decrease with serial passaging [Bonaventure et al., 1994], to fully develop only in long-term cultures [Binette et al., 1998; Liu et al., 1998], and to be highly dependent on the lot of serum used [Yaeger et al., 1997]. The ability of human expanded chondrocytes to redifferentiate was recently studied under controlled conditions in a chemically defined serum-free medium, and shown to be enhanced by specific combinations of growth factors and hormones during 3D culture [Yaeger et al., 1997]. However, the differentiation requirements for human chondrocytes expanded in the presence of different growth factors have not yet been investigated.

In this paper, we studied the effects of different growth factors and culture medium compositions on the coordinated expansion-redifferentiation of human articular chondrocytes by using a two-phase culture system (i.e., 2D monolayer culture and 3D cultivation in pellets). Based on previous works performed using chick [Quarto et al., 1997] and bovine calf [Martin et al., 1999] chondrocytes, our hypothesis was that expansion of adult human chondrocytes with specific growth factors can modulate not only their proliferation and differentiation, but also their ability to redifferentiate when transferred into a 3D environment. Growth factors supplemented to the culture medium during monolayer expansion were selected as known mitogens for chondrocytes [Guerne et al., 1995; Quarto et al., 1997], whereas factors used to redifferentiate cells in pellets were selected as known chondrogenic stimuli for dedifferentiated chondrocytes [Yaeger et al., 1997] or mesenchymal progenitor cells [Yoo et al., 1998]. In order to reliably quantify patterns of chondrocyte differentiation in a large number of culture conditions, the expression of collagen types I and II, as well as of aggrecan and versican, was assessed at the mRNA level using a recently developed method based on real time PCR [Martin et al., 2000].

## METHODS

### Culture of Human Articular Chondrocytes

**Cartilage specimens.** Human articular cartilage samples were collected from the hip or the ankle joints of four patients (mean age 57.5, range 25–73) with no history and no radiographic signs of joint disease, undergoing joint replacement following femoral neck fracture or foot amputation for tumor resection in the University Hospital of Basel.

**Phase I:** Cell isolation and expansion in monolayers. Chondrocytes were isolated upon 22-hour incubation at 37°C in 0.15% type II collagenase and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 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, and 0.29 mg/ml L-glutamine (control medium, CTR). The lot of serum used (40G2470J, Gibco BRL Life Technology) was selected among five different ones based on preliminary proliferation tests. Chondrocytes were plated on tissue culture flasks at approximately $10^4$ cells/cm$^2$ and cultured in a humidified 37°C/5% $CO_2$ incubator. After approximately 10 days, when cells were sub-confluent, first passage cells (P1) were detached using 0.25% trypsin/1 mM EDTA and replated at $5 \times 10^3$ cells/cm$^2$. After one more week, when cells again approached confluency, second passage (P2) cells were trypsinized and cultured in pellets as described below. Throughout the expansion phase, cells were cultured in CTR medium, or CTR medium supplemented with different growth factors, involved in cartilage metabolism. In particular, the following factors (all from R&D Systems, Minneapolis, MN) were tested: 5 ng/mL of fibroblast growth factor-2 (FGF-2), 10 ng/mL of epidermal growth factor (EGF), 10 ng/mL of platelet-derived growth factor-bb (PDGFbb), 1 ng/mL of transforming growth factor-β1 (TGF-β), or the combination of 5 ng/mL FGF-2

and 1 ng/mL TGF-β (FGF-2/TGFβ). The concentrations of these factors were selected based on preliminary dose-response studies. During expansion, chondrocytes underwent 5–7 doublings.

**Phase II: Cultivation of human chondrocytes in pellets.** P2 chondrocytes were suspended either in chemically defined serum-free medium (SFM) or in serum-supplemented medium (SSM). SFM consisted of DMEM supplemented with $ITS^{+1}$ (Sigma Chemical, St. Louis, MO; i.e., 10 μg/mL insulin, 5.5 mg/mL transferrin, 5 ng/mL selenium, 0.5 mg/mL bovine serum albumin, 4.7 mg/mL linoleic acid), 0.1 mM ascorbic acid 2-phosphate, and 1.25 mg/mL human serum albumin. The $ITS^{+1}$ supplements were selected based on previous reports that they were able to promote chondrogenic differentiation of human expanded chondrocytes [Yaeger et al., 1997] and mesenchymal progenitor cells [Yoo et al., 1998]. SSM was CTR medium supplemented with 10 μg/mL insulin and 0.1 mM ascorbic acid 2-phosphate. The concentration of insulin in SSM was the same as in the SFM and sufficient to substitute for the IGF-I requirement for human chondrocyte differentiation [Yaeger et al., 1997]. Aliquots of $5 \times 10^5$ cells in 0.5 mL of medium were centrifuged at 7500 rpm for 15 s in 1.5 mL polypropylene conical tubes (Sarstedt, Nümbrecht, D) to form spherical pellets, which were placed onto a 3D orbital shaker (Bioblock Scientific, Frenkendorf, CH) at 30 rpm in a humidified 37°C/5% $CO_2$ incubator. Pellets were cultured in four different medium compositions: SSM, SFM, SFM supplemented with 10 ng/mL TGF-β (SFM T), and SFM supplemented with T and $10^{-7}$ M dexamethasone (SFM TD). The concentrations of TGFβ and dexamethasone were selected based on previous studies on the differentiation of human chondrogenic cells [Yaeger et al., 1997; Yoo et al., 1998]. After 2 weeks, pellets were processed for histology, biochemistry, or mRNA analysis as described below.

## Analytical Methods

**Cell proliferation and differentiation in monolayers.** Doubling times and differentiation markers of P1 and P2 chondrocytes were obtained by plating primary and P1 chondrocytes, respectively, in 10 mm wells at the same density and in the same culture conditions described for cell expansion. DNA amounts were measured at timed intervals in triplicate using the CyQuant cell proliferation assay kit (Molecular Probes, Eugene, OR), according to the manufacturer's instructions. The doubling time of a cell population during the exponential growth phase was calculated as the ratio of T to $\log_2(N/N_0)$, where $N_0$ and $N$ were the DNA amounts at the beginning and end, respectively, of the exponential growth time (T) [Martin et al., 1999]. After the exponential growth phase, mRNA from sub-confluent wells was extracted and analyzed as described below.

**Histology and immunohistochemistry of cell pellets.** Cell pellets were rinsed with phosphate buffered solution (PBS), fixed in 4% buffered formalin for 24 h at 4°C, embedded in paraffin and cross-sectioned (5-μm thick). For histological evaluation, sections were stained with safranin O for sulfated glycosaminoglycans (GAG). For immunohistochemical analysis, sections were incubated for 1 h with 0.25% $NH_3$ in 70% ethanol during deparaffinization. Sections were then treated for 40 min on ice with 10 mg/mL $NaBH_4$, for 30 min at 37°C with 1 mg/mL testicular hyaluronidase, and for 15 min at room temperature (RT) with normal goat serum diluted 1:10 in PBS. Samples were labeled by incubation for 1 h at RT with a monoclonal antibody against collagen type II (II-II6B3, Developmental Studies Hybridoma Bank, Baltimore, MD), for 1 h at RT with Alexa 488 (Molecular Probes) diluted 1:800 and for 30 min at RT with 0.1% Sudan Black in 70% ethanol. $NH_3$, $NaBH_4$, and Sudan Black were used to reduce background fluorescence. Specimens were finally imaged by a confocal laser scanning microscope (TCS 4-D CLSM, Leica AG, Heidelberg) using Argon laser excitation and an Apochromat 25X 0.75 NA oil immersion objective. Optical sections were acquired at 0.5-μm increments and rendered using the Imaris software (Bitplane, Zürich, CH).

**Biochemical analysis of cell pellets.** Cell pellets 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 h at 56°C) [Hollander et al., 1994]. GAG contents were measured spectrophotometrically using dimethylmethylene blue [Farndale et al., 1986], with chondroitin sulfate as a standard, and normalized to the DNA amount, measured spectrofluorometrically using Hoechst 33258 [Kim et al., 1988], with calf thymus DNA as a standard.

**Total RNA extraction and cDNA synthesis**. RNA was extracted from expanded cells or pellets using Trizol (Life Technologies, Basel, CH) and the standard single-step acid-phenol guanidinium method [Chomczynski and Sacchi, 1987]. Pellets suspended in Trizol were first sonicated for 20 s. cDNA was generated from 2 μg of RNA by using murine MLV reverse transcriptase (BRL, Gaithersville, MD) in the presence of dNTPs and DTT, according to the manufacturer's instructions.

**Real-time PCR amplification and quantitative analysis**. PCR reactions were performed and monitored using a ABI Prism 7700 Sequence Detection System (Perkin–Elmer Applied Biosystems). The PCR master mix was based on AmpliTaq Gold DNA polymerase (Perkin–Elmer Applied Biosystems). cDNA samples (2.5 μL in a total volume of 25 μL per well) were analyzed in single (for cells in monolayers) or in duplicate (for cell pellets). Sequences of primers and probes for human GAPDH, collagen types I, II, and X, aggrecan and versican are described in [Martin et al., 2000]. After an initial denaturation step 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. Data analysis was carried out by using the Sequence Detector V program (Perkin–Elmer Applied Biosystems). For each sample, the Ct value was determined as the cycle number at which the fluorescence intensity reached 0.05; this value was chosen after confirming that, in this range, all curves were in the exponential phase of amplification. For each cDNA sample, the Ct value of each target sequence was subtracted from the Ct value of the reference gene (GAPDH), to derive ΔCt. The level of expression of each target gene was then calculated as $2^{\Delta Ct}$. This formula could be used since the efficiencies of amplification for the genes of interest and the housekeeping gene were comparable (<10% difference) and close to 100% [Martin et al., 2000]. Figures calculated as described above, however, cannot be considered as absolute expression levels of the different genes, and can only be used for relative comparisons among different culture conditions (PE-ABI; Sequence Detector User Bulletin 2).

Since collagen type II and aggrecan are the typical markers of differentiated chondrocytes in hyaline cartilage, as opposed to collagen type I and versican, which are expressed by dedifferentiated chondrocytes and in fibrocartilage, we used the ratios of mRNA levels of collagen type II to I (CII/CI) and of aggrecan to versican (Agg/Ver) as *differentiation indexes* related to the expression of collagens and proteoglycans, respectively [Martin et al., 2000].

**Statistical analysis.** Unless otherwise stated, all experiments were repeated at least three times with cells from different individuals. Values are presented as mean ± SD. Differences among experimental groups were assessed by one-way analysis of variance (ANOVA). Values of $P < 0.05$ were considered to indicate statistically significant differences.

## RESULTS

### Effects of Different Growth Factors on Chondrocyte Expansion

All the growth factors tested during chondrocyte expansion in monolayers induced a significant decrease in the doubling times of exponential growth, with the exception of EGF during P1 (Table IA). During P1, FGF-2 and TGFβ promoted the highest cell proliferation rates, and the combination of the two factors had an additive effect, reducing the doubling times of CTR cultures by about 40%. During P2, chondrocytes proliferated generally faster than during P1, except when stimulated by TGFβ. Cells cultured in the presence of FGF-2/TGFβ displayed the lowest doubling times, averaging about 50% of the CTR condition.

Ratios of collagen type II to I (CII/CI) and of aggrecan to versican (Agg/Ver) at the mRNA level averaged, respectively, $288 \pm 115$ and $90 \pm 25$ in native cartilage samples used for this study. Both indexes were markedly lower in P1 cells in all culture conditions, confirming that chondrocyte expansion was associated with the loss of the differentiated phenotype (Table IB,C). After P1, CII/CI and Agg/Ver values were significantly lower (up to 100 fold) in chondrocytes expanded in the presence of FGF-2, TGFβ and FGF-2/TGFβ than in CTR cells. After P2, CII/CI ratios further decreased in all culture conditions and were the lowest if cells were cultivated in the presence of FGF-2/TGFβ (more than 500-fold lower than in CTR cultures). Agg/Ver ratios after P2 were comparable to those measured after P1, in all culture conditions. As compared to CTR cells, the expression of

**TABLE I. Chondrocyte Proliferation and Differentiation During Monolayer Expansion[a]**

| | | Expansion Condition | | | | | |
|---|---|---|---|---|---|---|---|
| | | CTR | EGF | PDGFbb | FGF-2 | TGFβ | FGF-2/TGFβ |
| **A** | Doubling time (hours) | | | | | | |
| | During P1 | 84.4 ± 10.9 | 71.4 ± 7.8 | 63.9 ± 6.6[b] | 59.1 ± 4.5[b] | 59.0 ± 7.1[b] | 49.8 ± 1.0[c] |
| | During P2 | 76.6 ± 3.9 | 67.1 ± 1.7[*] | 47.2 ± 1.4[b] | 47.7 ± 2.0[b] | 66.2 ± 4.2[b] | 38.2 ± 2.9[c] |
| **B** | CII/CI mRNA ratio | | | | | | |
| | After P1 | 1.16 ± 0.40 | 0.93 ± 0.24 | 1.47 ± 0.20 | 0.35 ± 0.14[b] | 0.08 ± 0.05[b] | 0.01 ± 0.00[c] |
| | After P2 | 0.86 ± 0.58 | 0.26 ± 0.12[b] | 0.23 ± 0.12[b] | 0.20 ± 0.06[b] | 0.03 ± 0.02[b] | 0.00 ± 0.00[c] |
| **C** | Agg/Ver mRNA ratio | | | | | | |
| | After P1 | 1.50 ± 0.57 | 2.52 ± 1.53 | 1.57 ± 0.75 | 0.46 ± 0.10[b] | 0.06 ± 0.02[b] | 0.08 ± 0.03[b] |
| | After P2 | 2.81 ± 1.04 | 1.83 ± 0.93 | 1.47 ± 0.43 | 0.40 ± 0.21[b] | 0.19 ± 0.11[b] | 0.44 ± 0.31[b] |
| **D** | CI mRNA | | | | | | |
| | After P1 | 1.82 ± 0.13 | 2.91 ± 1.04 | 2.16 ± 0.60 | 0.69 ± 0.22[b] | 17.97 ± 0.57[c] | 7.86 ± 2.22[b] |
| | After P2 | 2.46 ± 1.37 | 3.45 ± 0.19 | 3.87 ± 1.15 | 1.32 ± 0.25 | 31.72 ± 5.22[c] | 7.25 ± 2.42[b] |
| **E** | Ver mRNA | | | | | | |
| | After P1 | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.02 ± 0.01 | 0.04 ± 0.01[b] | 0.08 ± 0.01[b] | 0.14 ± 0.05[b] |
| | After P2 | 0.02 ± 0.01 | 0.01 ± 0.00 | 0.02 ± 0.01 | 0.06 ± 0.02[b] | 0.06 ± 0.02[b] | 0.11 ± 0.04[b] |

[a]See the Methods section for definition of abbreviations.
[b]Statistically significant difference from the CTR expansion condition.
[c]Statistically significant difference from all other expansion conditions.

collagen type I was lower in chondrocytes expanded with FGF-2, and higher if cells were expanded with TGFβ or FGF-2/TGFβ (Table ID). Therefore, as compared to the CTR condition, CII/CI ratios were lower in FGF-2 expanded chondrocytes mainly due to a decrease in CII expression, whereas in TGFβ expanded chondrocytes the same effect was mainly due to an increase in CI expression. In FGF-2/TGFβ expanded cells, CII/CI ratios were the lowest due to the combined up- and down-regulation of CI and CII, respectively (Table IB,D). As compared to CTR cultures, lower Agg/Ver ratios in FGF-2, TGFβ and FGF-2/TGFβ expanded cells were always due to a combined up- and down-regulation of versican and aggrecan, respectively (Table IC,E).

Morphological observations matched molecular data regarding chondrocyte differentiation. As an example, Figure 1 illustrates that after P1 cells expanded in CTR medium had still a round shape, characteristic of the differentiated chondrocyte phenotype (Fig. 1A), whereas cells expanded with FGF-2/TGFβ displayed the typical fibroblast-like phenotype of dedifferentiated chondrocytes (Fig. 1B).

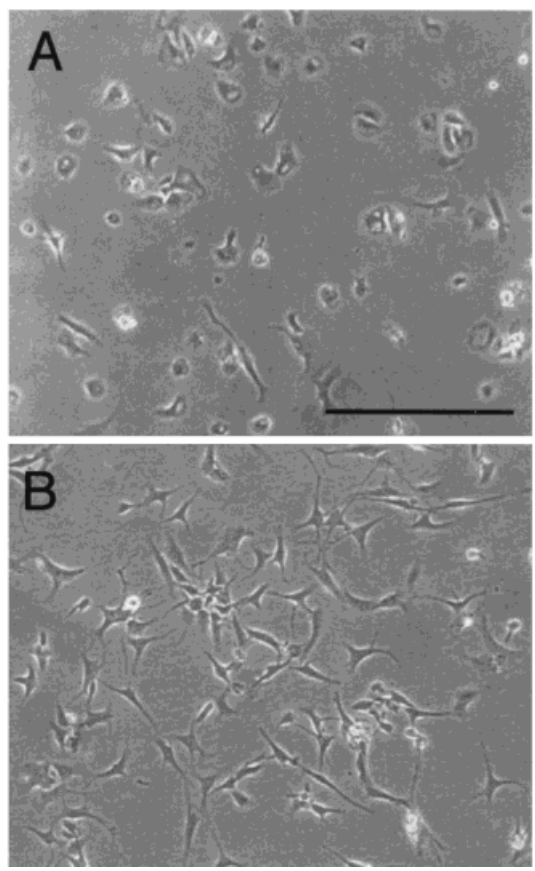


**Fig. 1.** Comparative morphology of chondrocytes cultured for 1 week in CTR medium (**A**) or in the presence of FGF-2/TGFβ (**B**). Chondrocytes expanded in CTR medium retained a rounded morphology, whereas FGF-2/TGFβ-expanded cells appeared more elongated and fibroblast-like. Scale bar = 100 μm.

### Redifferentiation of Expanded Chondrocytes in Cell Pellets

The redifferentiation potential of P2 chondrocytes expanded with the different growth factors was evaluated in pellets cultured under

**TABLE II. Chondrocyte Differentiation in Pellet Cultures[a]**

| | | Expansion Condition | | | | | |
|---|---|---|---|---|---|---|---|
| | | CTR | EGF | PDGFbb | FGF-2 | TGFβ | FGF-2/TGFβ |
| **A** | CII/CI mRNA ratio | | | | | | |
| | Pellets in SSM | $0.20 \pm 0.16$ | $0.03 \pm 0.02$ | $0.03 \pm 0.01$ | $0.08 \pm 0.04$ | $0.02 \pm 0.01^{b}$ | $0.02 \pm 0.01^{b}$ |
| | Pellets in SFM | $0.49 \pm 0.22$ | $0.35 \pm 0.18$ | $0.48 \pm 0.25^{b}$ | $0.46 \pm 0.26$ | $0.04 \pm 0.02^{b}$ | $0.01 \pm 0.00^{c}$ |
| | Pellets in SFM T | $0.58 \pm 0.32$ | $0.07 \pm 0.05^{b}$ | $0.05 \pm 0.03^{b}$ | $0.39 \pm 0.25$ | $0.02 \pm 0.01^{b}$ | $0.16 \pm 0.04$ |
| | Pellets in SFM TD | $0.52 \pm 0.23$ | $0.08 \pm 0.03^{b}$ | $0.11 \pm 0.08^{b}$ | $0.45 \pm 0.19$ | $0.13 \pm 0.04^{b}$ | $0.96 \pm 0.10^{c}$ |
| **B** | Agg/Ver mRNA ratio | | | | | | |
| | Pellets in SSM | $4.88 \pm 1.70^{c}$ | $0.91 \pm 0.67^{b}$ | $1.09 \pm 0.72^{b}$ | $1.03 \pm 0.26^{b}$ | $1.37 \pm 0.37^{b}$ | $0.69 \pm 0.26^{b}$ |
| | Pellets in SFM | $3.66 \pm 2.13$ | $0.78 \pm 0.52$ | $0.75 \pm 0.39$ | $5.02 \pm 2.44$ | $1.18 \pm 0.72$ | $0.26 \pm 0.17^{b}$ |
| | Pellets in SFM T | $0.31 \pm 0.04$ | $0.07 \pm 0.03^{b}$ | $0.17 \pm 0.06^{b}$ | $1.50 \pm 1.12$ | $0.20 \pm 0.08$ | $1.18 \pm 0.79$ |
| | Pellets in SFM TD | $0.43 \pm 0.13$ | $0.19 \pm 0.10$ | $0.21 \pm 0.12$ | $2.09 \pm 0.87^{b}$ | $0.38 \pm 0.16$ | $12.07 \pm 1.83^{c}$ |
| **C** | CII mRNA | | | | | | |
| | Pellets in SFM TD | $7.59 \pm 2.08$ | $0.94 \pm 0.07^{b}$ | $4.52 \pm 1.60$ | $4.52 \pm 1.94$ | $1.34 \pm 0.73^{b}$ | $25.55 \pm 3.00^{c}$ |
| **D** | Agg mRNA | | | | | | |
| | Pellets in SFM TD | $0.03 \pm 0.02$ | $0.01 \pm 0.00$ | $0.05 \pm 0.02$ | $0.25 \pm 0.13^{b}$ | $0.18 \pm 0.08^{b}$ | $0.31 \pm 0.09^{b}$ |

[a]See the Methods for definition of abbreviations.
[b]Statistically significant difference from the CTR expansion condition.
[c]Statistically significant difference from all other expansion conditions.

different culture medium compositions. After 2 weeks cultivation in a serum-supplemented medium (SSM), CII/CI and Agg/Ver mRNA ratios were highest in pellets based on CTR expanded chondrocytes (Table IIA,B). However, ratios were not significantly different from those measured in P2 cells, before they were transferred into the 3D environment (Table IB,C), indicating that a real redifferentiation did not occur. CII/CI ratios significantly increased during the 3D culture phase only in pellets based on FGF-2/TGFβ-expanded chondrocytes (Tables IB and IIA). Pellets cultured in serum-free medium (SFM) had generally higher CII/CI levels than those cultured in SSM, unless chondrocytes were previously cultured in FGF-2/TGFβ supplemented medium (Table IIA). Agg/Ver ratios in pellets cultured in SFM were comparable to those of SSM cultures, except for pellets based on FGF-2-expanded chondrocytes, which displayed significantly increased values (Table IIB). Upon supplementation of the SFM with TGFβ (SFM T), CII/CI ratios were not affected in pellets based on CTR, FGF-2 and TGFβ expanded chondrocytes, significantly decreased in pellets based on EGF- and PDGFbb-expanded cells, and significantly increased in pellets based on FGF-2/TGFβ expanded chondrocytes (Table IIA). Agg/Ver ratios decreased by supplementation of SFM with TGFβ, unless cells were previously expanded with FGF-2/TGFβ (Table IIB). Further supplementation of dexamethasone to the SFM T (SFM TD) significantly increased the CII/CI and Agg/Ver ratios only in pellets based on cells expanded with FGF-2/TGFβ (Table IIA,B). Cells expanded with FGF-2/TGFβ and cultured as pellets in SFM TD displayed the highest CII/CI and Agg/Ver ratios of all groups (respectively, 1.8- and 28.1-fold higher than in the corresponding CTR group) and expressed the largest amounts of collagen type II and aggrecan genes (respectively, 3.3- and 10.3-fold higher than in the corresponding CTR group) (Table IIC,D). Supplementation of SFM with dexamethasone without TGFβ did not result in changes in the CII/CI and Agg/Ver mRNA ratios in pellets based on FGF-2/TGFβ expanded cells (data not shown). The expression of collagen type X gene at the mRNA level was negligible in all pellets, and did not appear to be modulated by the culture medium composition.

The GAG/DNA content of selected pellets after 2 weeks culture is presented in Figure 2. Chondrocytes expanded in CTR and FGF-2/TGFβ medium and cultured as pellets in SSM produced comparable, low amounts of GAG. The GAG/DNA content was significantly higher when pellets were cultured in SFM TD than when cultured in SSM, and was the highest if pellets were based on FGF-2/TGFβ-expanded chondrocytes. Pellets cultured in SSM appeared histologically as clusters of elongated cells, without deposition of cartilaginous extracellular matrix (Fig. 3A,B). Pellets based on CTR expanded cells and cultured in SFM TD

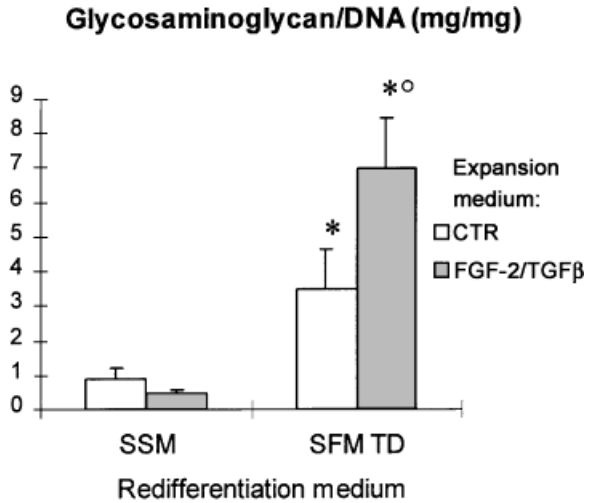


**Fig. 2.** Glycosaminoglycan (GAG) content normalized to the DNA amount of selected pellets. Pellets were based on chondrocytes expanded in CTR medium (white bars) or in the presence of FGF-2/TGFβ (gray bars) and were cultured for 2 weeks in serum-supplemented medium (SSM) or in serum-free medium supplemented with TGFβ and dexamethasone (SFM TD). An asterisk (*) indicates statistically significant difference from the pellets generated by the same cells and cultured in SSM; an empty circle (○) indicates statistically significant difference from the pellets generated by CTR cells and cultured in the same medium.

displayed sparse foci of extracellular matrix containing sulfated GAG, as assessed by safranin O stain (Fig. 3C), and contained negligible amounts of collagen type II, as assessed immunohistochemically (Fig. 4A). Pellets based on FGF-2/TGFβ-expanded cells and cultured in SFM TD accumulated a continuous extracellular matrix which was uniformly stained for GAG (Fig. 3D) and type II collagen (Fig. 4B), although signs of interterritorial matrix and cell lacunae were still not evident.

## DISCUSSION

In this paper, we demonstrated that the conditions of expansion of human articular chondrocytes can modulate the cell ability to re-enter the differentiation program upon transfer into a 3D environment. Moreover, we showed that chondrocytes expanded in the presence of FGF-2/TGFβ displayed not only the highest proliferation rate and the most enhanced dedifferentiation in monolayers, but also the highest capacity to redifferentiate and generate a cartilaginous tissue in response to TGFβ and dexamethasone, supplemented during the 3D culture in pellets.

The markers of differentiation used in this study (i.e., CII/CI and Agg/Ver) have been quantified in monolayer and pellet cultures using recently developed real-time PCR assays [Martin et al., 2000]. Real-time PCR enables quantification of mRNA with a high accuracy, reproducibility and sensitivity in a wide dynamic range, without the need of post-PCR processing [Gibson et al., 1996], and was essential to reliably evaluate different combinations of factors used during the expansion and redifferentiation phases. The defined differentiation characteristics at the mRNA level were in agreement with those at the protein level, as assessed in selected conditions using biochemical, histological, and immunohistochemical techniques.

The differential effects of several growth factors on the proliferation and differentiation of monolayer cultures of adult human articular chondrocytes have been systematically quantified during the first and second passages of expansion by measuring DNA amounts at defined time intervals and by real-time PCR. Our results support the concept that factors stimulating the highest chondrocyte proliferation also induce the strongest cell dedifferentiation. Furthermore, they provide evidence that the proliferative/dedifferentiating effects of FGF-2 and TGFβ on human primary chondrocytes cultured in monolayers are additive, as previously reported for avian [Horton et al., 1989] and rodent [Bradham and Horton, 1998] chondrocytes.

The redifferentiation potential of human chondrocytes expanded with different growth factors has been studied in 3D pellets using different culture medium compositions. As compared to a serum-supplemented medium (SSM), the use of a defined serum-free medium (SFM) supported a comparable extent of differentiation in chondrocytes expanded in the CTR medium, in agreement with previous reports [Yaeger et al., 1997], but significantly enhanced the redifferentiation of chondrocytes expanded with EGF, PDGFbb, or FGF-2. These results could suggest the presence in serum of differentiation inhibitors, only affecting cells expanded in the presence of specific growth factors. We also report that supplementation of SFM with TGFβ, alone or in combination with dexamethasone, markedly increased the CII/CI mRNA ratio of FGF-2/TGFβ-expanded chondrocytes, but reduced that of EGF- and PDGFbb-expanded cells. Taken together, our

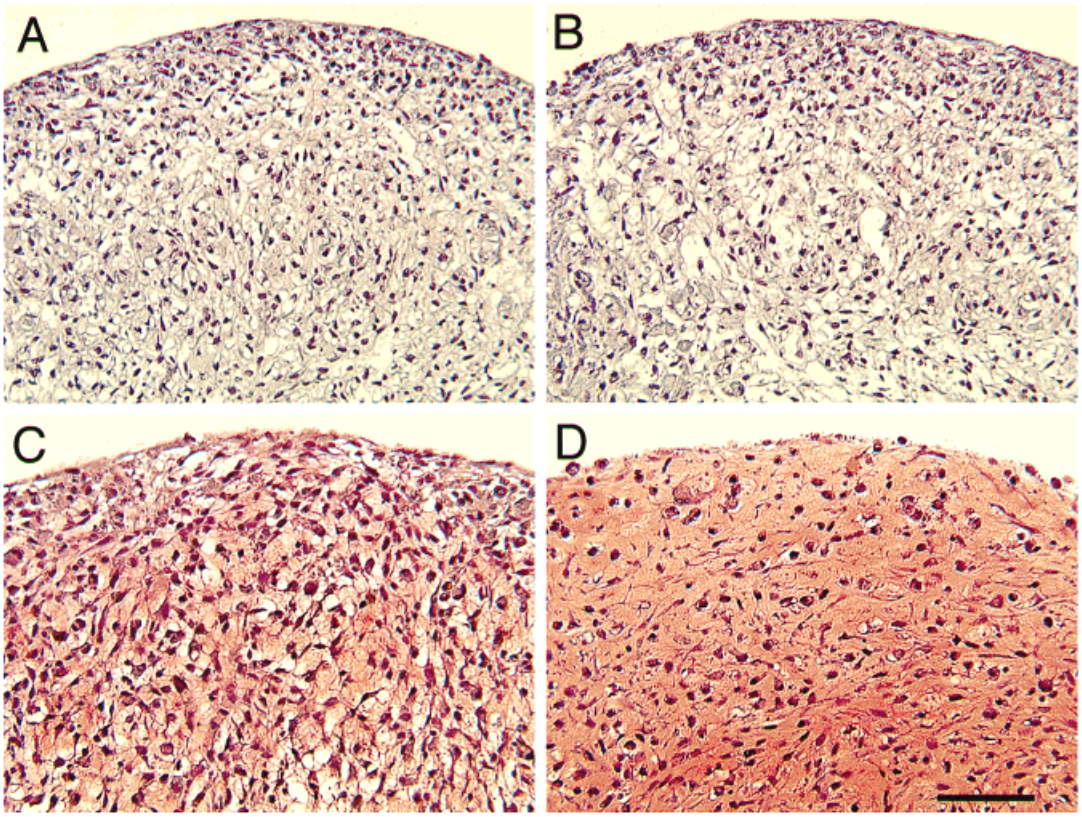


**Fig. 3.** Histological appearance of chondrocyte pellets. Chondrocytes expanded in CTR medium (**A**,**C**) or in the presence of FGF-2/TGFβ (**B**,**D**) were cultured for 2 weeks in serum-supplemented medium (SSM; **A**,**B**) or in serum-free medium supplemented with TGFβ and dexamethasone (SFM TD; **C**, **D**) in the form of pellets. Sections were stained with Safranin O for glycosaminoglycans (GAG). Pellets cultured in SSM did not contain extracellular matrix (ECM) with detectable amounts of GAG (**A**,**B**). Pellets cultured in SFM TD displayed scattered areas of ECM containing GAG if based on chondrocytes expanded in CTR medium (**C**), and consisted of a continuous ECM rich in GAG if based on chondrocytes expanded with FGF-2/TGFβ (**D**). Scale bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

data demonstrate that the conditions of chondrocyte expansion in monolayers modulate at the transcriptional level their commitment to redifferentiation and their ability to respond to regulatory molecules in a 3D environment.

The effects of TGFβ on chondrocytes have previously been proposed to depend on the context in which the factor is provided [Trippel, 1995]. In particular, TGFβ was unable to induce expression of chondrocytic markers in monolayer cultures [Galera et al., 1992], but had a chondrogenic effect on chondrocytes cultured in suspension [Harrison et al., 1992] and in alginate gels [Yaeger et al., 1997]. Therefore, it was postulated that the effects induced by TGFβ are related to the ability of cells to acquire a spherical morphology during stimulation [Yaeger et al., 1997]. Our results indicating that TGFβ stimulates chondrocyte dedifferentiation in monolayers and may induce cell redifferentiation in pellet cultures are consistent with the previously reported observations. However, the fact that TGFβ supplementation reduced the CII/CI and Agg/Ver mRNA ratios of EGF- and PDGFbb-expanded chondrocytes while it increased the same differentiation markers in cells expanded with FGF-2/TGFβ suggests that the effects of TGFβ are also dependent on the history of cell expansion and/or the stage of cell differentiation.

Chondrocytes expanded in the presence of FGF-2/TGFβ displayed the lowest CII/CI ratios after P1 and P2, but were able to reach the highest differentiation levels and to generate the best cartilaginous tissues when cultured in 3D pellets. Our findings demonstrate that the efficiency of chondrocyte redifferentiation is not inversely proportional to the time the cells remain dedifferentiated during expansion, as previously proposed [Benya and Shaffer, 1982].

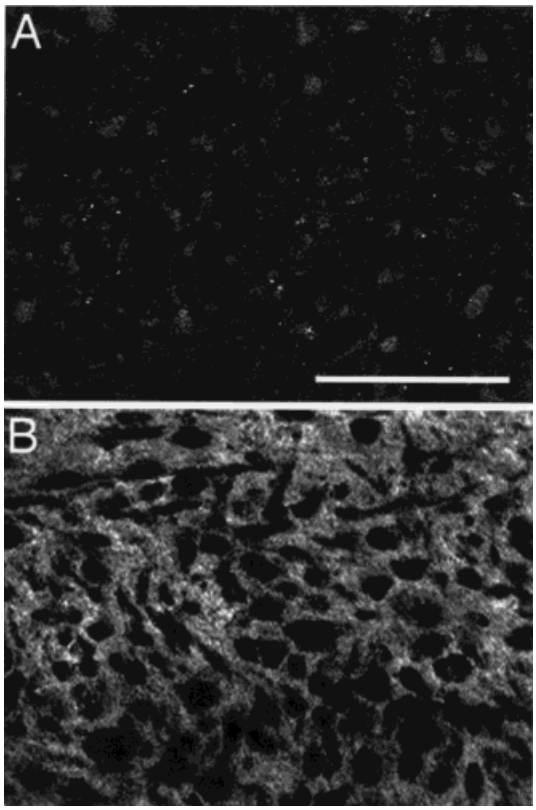


**Fig. 4.** Collagen type II immunostain of pellets based on chondrocytes expanded in CTR medium (**A**) or in the presence of FGF-2/TGFβ (**B**) and cultured for 2 weeks in serum-free medium supplemented with TGFβ and dexamethasone (SFM TD). Chondrocytes expanded in CTR medium accumulated negligible amounts of collagen type II, whereas FGF-2/TGFβ-expanded chondrocytes deposited an extracellular matrix continuously stained for collagen type II. Confocal microscopy images, scale bar = 50 μm.

It will be interesting to test whether other factors, possibly related to the cytoskeletal organization [Martin et al., 1999] or to the expression of specific adhesion molecules/surface markers [Chimal-Monroy and Diaz, 1999], are able to predict the redifferentiation potential of expanded chondrocytes. FGF-2/TGFβ-expanded chondrocytes redifferentiated only if stimulated with TGFβ and dexamethasone, a medium composition previously shown to induce chondrogenic differentiation of human mesenchymal progenitor cells from the bone marrow [Yoo et al., 1998]. It would be tempting to speculate that medium supplementation with the combination of FGF-2 and TGFβ during chondrocyte monolayer cultures supports the expansion of a cell lineage with similar features to mesenchymal progenitor cells. Under our experimental conditions, however, FGF-2/TGFβ-expanded chondrocytes did not undergo hyperthrophy, as assessed by the negligible expression of collagen type X. This result is consistent with the hypothesis that the chondrogenic potential of dedifferentiated articular chondrocytes has distinct features from that of bone marrow stromal cells, epiphyseal growth plate chondrocytes, or periosteal cells [Binette et al., 1998].

Our study represents a relevant step toward the engineering of autologous cartilaginous grafts starting from small tissue biopsies, as an extension of previous findings based on calf bovine chondrocytes [Martin et al., 1999]. Expansion of adult human articular chondrocytes with FGF-2/TGFβ would allow not only faster cell amplification and/or reduction of the required amount of donor tissue, but also better maintainance of the cell ability to differentiate and produce cartilage-specific extracellular matrix in response to chondrogenic stimuli. Studies are under way in our laboratory to evaluate the potential of culture expanded human chondrocytes to generate cartilaginous tissues of predefined size and shape by using appropriate regulatory molecules in conjunction with specific 3D structural templates.

## ACKNOWLEDGMENTS

We are indebted to Rosemarie Sütterlin and Werner Baschong for the collagen type II immunostainings, to Michael Stumm for the histological assistance, and to Giulio Spagnoli for a critical reading of the manuscript.

## REFERENCES

Benya PD, Shaffer JD. 1982. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 30:215–224.

Binette F, McQuaid DP, Haudenschild DR, Yaeger PC, McPherson JM, Tubo R. 1998. Expression of a stable articular cartilage phenotype without evidence of hypertrophy by adult human articular chondrocytes in vitro. J Orthop Res 16:207–216.

Bonaventure J, Kadhom N, Cohen-Solal L, Ng KH, Bourguignon J, Lasselin C, Freisinger P. 1994. Reexpression of cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. Exp Cell Res 212:97–104.

Bradham DM, Horton WEJ. 1998. In vivo cartilage formation from growth factor modulated articular chondrocytes. Clin Orthop 239–249.

Chimal-Monroy J, Diaz dL. 1999. Expression of N-cadherin, N-CAM, fibronectin and tenascin is stimulated by TGF-beta1, beta2, beta3 and beta5 during the

formation of precartilage condensations. Int J Dev Biol 43:59–67.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. Anal Biochem 162:156–159.

de Haart M, Marijnissen WJ, van Osch GJ, Verhaar JA. 1999. Optimization of chondrocyte expansion in culture. Effect of TGF beta-2, bFGF and L-ascorbic acid on bovine articular chondrocytes. Acta Orthop Scand 70:55–61.

Evans CH, Georgescu HI. 1983. Observations on the senescence of cells derived from articular cartilage. Mech Ageing Dev 22:179–191.

Farndale RW, Buttle DJ, Barrett AJ. 1986. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 883:173-177.

Freed LE, Grande DA, Lingbin Z, Emmanual J, Marquis JC, Langer R. 1994. Joint resurfacing using allograft chondrocytes and synthetic biodegradable polymer scaffolds. J Biomed Mater Res 28:891–899.

Galera P, Redini F, Vivien D, Bonaventure J, Penfornis H, Loyau G, Pujol JP. 1992. Effect of transforming growth factor-beta 1 (TGF-beta 1) on matrix synthesis by monolayer cultures of rabbit articular chondrocytes during the dedifferentiation process. Exp Cell Res 200:379–392.

Gibson UE, Heid CA, Williams PM. 1996. A novel method for real time quantitative RT-PCR. Genome Res 6:995-1001.

Guerne PA, Blanco F, Kaelin A, Desgeorges A, Lotz M. 1995. Growth factor responsiveness of human articular chondrocytes in aging and development. Arthritis Rheum 38:960–968.

Harrison ETJ, Luyten FP, Reddi AH. 1992. Transforming growth factor-beta: its effect on phenotype reexpression by dedifferentiated chondrocytes in the presence and absence of osteogenin. In Vitro Cell Dev Biol 28A:445–448.

Hollander AP, Heathfield TF, Webber C, Iwata Y, Bourne R, Rorabeck C, Poole AR. 1994. Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay. J Clin Invest 93:1722–1732.

Horton WEJ, Higginbotham JD, Chandrasekhar S. 1989. Transforming growth factor-beta and fibroblast growth factor act synergistically to inhibit collagen II synthesis through a mechanism involving regulatory DNA sequences. J Cell Physiol 141:8–15.

Kawamura S, Wakitani S, Kimura T, Maeda A, Caplan AI, Shino K, Ochi T. 1998. Articular cartilage repair. Rabbit experiments with a collagen gel-biomatrix and chondrocytes cultured in it. Acta Orthop Scand 69:56–62.

Kim YJ, Sah RL, Doong JY, Grodzinsky AJ. 1988. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. Anal Biochem 174:168–176.

Liu H, Lee YW, Dean MF. 1998. Re-expression of differentiated proteoglycan phenotype by dedifferentiated human chondrocytes during culture in alginate beads. Biochim Biophys Acta 1425:505–515.

Martin I, Jakob M, Schäfer D, Dick W, Spagnoli G, and Heberer, M. (2000). Quantitative analysis of gene expression in human articular cartilage from normal and osteoarthritic joints. Osteoarthritis Cartilage (in press).

Martin I, Vunjak-Novakovic G, Yang J, Langer R, Freed LE. 1999. Mammalian chondrocytes expanded in the presence of fibroblast growth factor 2 maintain the ability to differentiate and regenerate three-dimensional cartilaginous tissue. Exp Cell Res 253:681–688.

Quarto R, Campanile G, Cancedda R, Dozin B. 1997. Modulation of commitment, proliferation, and differentiation of chondrogenic cells in defined culture medium. Endocrinology 138:4966–4976.

Sittinger M, Perka C, Schultz O, Haupl T, Burmester GR. 1999. Joint cartilage regeneration by tissue engineering. Z Rheumatol 58:130–135.

Stewart MC, Saunders KM, Burton-Wurster N, Macleod JN. 2000. Phenotypic stability of articular chondrocytes in vitro: the effects of culture models, bone morphogenetic protein 2, and serum supplementation. J Bone Miner Res 15:166–174.

Trippel SB. 1995. Growth factor actions on articular cartilage. J Rheumatol Suppl 43:129-32:129–132.

Vivien D, Galera P, Lebrun E, Loyau G, Pujol JP. 1990. Differential effects of transforming growth factor-beta and epidermal growth factor on the cell cycle of cultured rabbit articular chondrocytes. J Cell Physiol 143:534–545.

Yaeger PC, Masi TL, de Ortiz JL, Binette F, Tubo R, McPherson JM. 1997. Synergistic action of transforming growth factor-beta and insulin-like growth factor-I induces expression of type II collagen and aggrecan genes in adult human articular chondrocytes. Exp Cell Res 237:318–325.

Yoo JU, Barthel TS, Nishimura K, Solchaga L, Caplan AI, Goldberg VM, Johnstone B. 1998. The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. J Bone Joint Surg Am 80:1745–1757.