Chondrocytes Expressing Intracellular Collagen Type II Enter the Cell Cycle and Co-Express Collagen Type I in Monolayer Culture

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ABSTRACT: For autologous chondrocyte transplantation, articular chondrocytes are harvested from cartilage tissue and expanded in vitro in monolayer culture. We aimed to characterize with a cellular resolution the synthesis of collagen type II (COL2) and collagen type I (COL1) during expansion in order to further understand why these cells lose the potential to form cartilage tissue when re-introduced into a microenvironment that supports chondrogenesis. During expansion for six passages, levels of transcripts encoding COL2 decreased to <0.1%, whereas transcript levels encoding COL1 increased 370-fold as compared to primary chondrocytes. Flow cytometry for intracellular proteins revealed that chondrocytes acquired a COL2/COL1-double positive phenotype during expansion, and the COL2 positive cells were able to enter the cell cycle. While the fraction of COL2 positive cells decreased from 70% to <2% in primary chondrocytes to passage six cells, the fraction of COL1 positive cells increased from <1% to >95%. In parallel to the decrease of the fraction of COL2 positive cells, the cells' potential to form cartilage-like tissue in pellet cultures steadily decreased. Intracellular staining for COL2 enables for characterization of chondrocyte lineage cells in more detail with a cellular resolution, and it may allow predicting the effectiveness of expanded chondrocytes to form cartilage-like tissue. © 2014 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 32:1503–1511, 2014.

Keywords: chondrocyte; collagen type II; flow cytometry; bromodeoxyuridine; monolayer culture

For more than two centuries, it is known that traumatic defects of the articular cartilage do not heal spontaneously.[1] Therapeutic interventions for the treatment of these defects aim to delay or prevent progression to osteoarthritis. More than twenty years ago, a promising cell-based repair strategy known as autologous chondrocyte transplantation (ACT) was introduced in clinics.[2,3] During ACT, chondrocytes are extracted from their native environment of cartilage tissue and grown in monolayer cultures for amplification in order to obtain the number of cells needed for therapy.[3] During the adaptation to environmental changes from cartilage tissue to the tissue culture plate, the chondrocytes begin to proliferate and acquire a fibroblast-like phenotype with decreased expression of collagen type II (COL2) and increased expression of collagen type I (COL1).[4,5] When these cells are subsequently transferred into an environment favoring chondrogenesis, such as in vivo into a cartilage defect during ACT or in vitro into a pellet culture systems that mimic the events of chondrogenesis and cartilage formation,[4] they have typically lost the capacity to form hyaline cartilage but rather produce a fibrocartilaginous tissue.[6,7]

The synthesis of COL2 is characteristic of cells of the chondrocyte lineage, and COL2 is the main constituent of the cartilage matrix. COL2 is composed of three alpha-1 chains forming a triple helix structure, and during posttranslational processing, proline residues are hydroxylated by the proline-4-hydroxylase enzyme, using ascorbic acid as an essential co-factor.[8] The procollagen is then exported to the extracellular domain, the non-helical C- and N-terminal ends are cleaved, and through polymerization, collagen fibrils and fibers are formed. In addition to being a major structural component of cartilage tissue, extracellular COL2 has been shown to act in a chondroinstructive manner on chondrocyte lineage cells through interaction with integrin receptors on the cell surface.[9,10] The importance of COL2 in cartilage and chondrocytes is evident, and as such monitoring the expression of COL2 is a critical parameter to assess chondrocyte lineage cells. The purpose of this study was to characterize with a cellular resolution the phenotypic adaptations that articular chondrocytes undergo in monolayer culture after harvesting from cartilage tissue, with particular focus on the expression of COL2 and COL1 proteins and to further understand why these cells lose the potential to form cartilage tissue when transferred into a microenvironment that supports chondrogenesis.

MATERIALS AND METHODS

Expansion of Primary Chondrocytes

Bovine articular cartilage was harvested from the humeral heads of 2-year-old animals obtained within 24 h after death. Chondrocytes were isolated by sequential enzymatic digestion of the cartilage with 2.5 mg/ml pronase (Sigma–Aldrich, Buchs, Switzerland) for 1 h and 450 μg/ml collagenase P (Roche Diagnostics, Rotkreuz, Switzerland) for 4 h at 37°C. The released cells were expanded in proliferation medium (DMEM/Ham's F12 [Gibco, Life Technologies, Zug, Switzerland]) containing 10% fetal bovine serum (FBS, Sigma–Aldrich) and penicillin/streptomycin (P/S, 100 units/ml and 100 μg/ml, respectively, Gibco). Near confluence, the cells were released with Trypsin/EDTA (Gibco) and re-plated until reaching passage 6 (P6). Twenty-four hours after isolation and then at

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Table 1. Characterization of Expanded Bovine Articular Chondrocytes

Passage	Population Doublings	Levels of Transcripts (%)				
		COL2A1	ACAN	PG4	COL1A1	VCAN
PC		100 ± 7.5 (923 ± 69)	100 ± 8.3 (176 ± 14)	100 ± 33.3 (3.83 ± 1.27)	100 ± 67.7 (0.76 ± 0.14)	100 ± 8.0 (0.22 ± 0.03)
P1	2.5 ± 0.3	*10.9 ± 6.3*	*25.0 ± 21.2*	*22.0 ± 8.4*	*20,549 ± 7,362*	427 ± 177
P2	5.9 ± 0.9	*2.0 ± 1.2*	*8.5 ± 5.3*	*6.75 ± 6.7*	*33,976 ± 10,153*	499 ± 269
P3	10.1 ± 2.1	*0.5 ± 0.5*	*6.2 ± 7.4*	*1.5 ± 1.3*	*34,248 ± 4,894*	524 ± 256
P4	14.2 ± 3.2	*0.2 ± 0.2*	*4.1 ± 4.0*	*0.5 ± 0.4*	*30,357 ± 1,009*	449 ± 166
P5	17.9 ± 4.5	*0.1 ± 0.1*	*3.4 ± 2.8*	*0.3 ± 0.2*	*35,234 ± 5,166*	393 ± 126
P6	21.8 ± 5.3	*0.04 ± 0.02*	*3.0 ± 1.9*	*0.5 ± 0.6*	*37,246 ± 13,247*	374 ± 104

Levels of transcripts coding for COL2A1, ACAN, and COL1A1 were measured and normalized to primary chondrocytes (PC). For primary chondrocytes, the expression of these genes relative to B2M is shown in brackets. Values are mean ± standard deviation (SD) of three animals. Values which are significantly different ($p < 0.05$) as compared to primary chondrocytes are written in italics.

each passage, cells were cryopreserved and stored in liquid nitrogen for further experiments.

Cartilage Formation in Pellet Cultures

Pellet cultures were performed by transferring 5×10^5 cells in DMEM containing P/S, ITS^{+3} (Sigma–Aldrich), 0.1 mM ascorbic acid-2-phosphate (Sigma–Aldrich), 0.4 mM L-proline (Sigma–Aldrich), 100 nM dexamethasone (Sigma–Aldrich) to 15 ml polypropylene tubes and subsequently centrifuging at 250*g* for 5 min.[11] Cultures were stopped after 3 weeks.

Analysis of Gene Expression

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. Pellet cultures were first digested using 450 μg/ml collagenase P for 3 h at 37°C to release the chondrocytes from the matrix. Cells were then washed and subjected to the standard protocol for RNA extraction. RNA was reverse transcribed using MLV reverse transcriptase (Promega, Dübendorf, Switzerland). Real-time PCR was performed using TaqMan® assays according to the manufacturer's

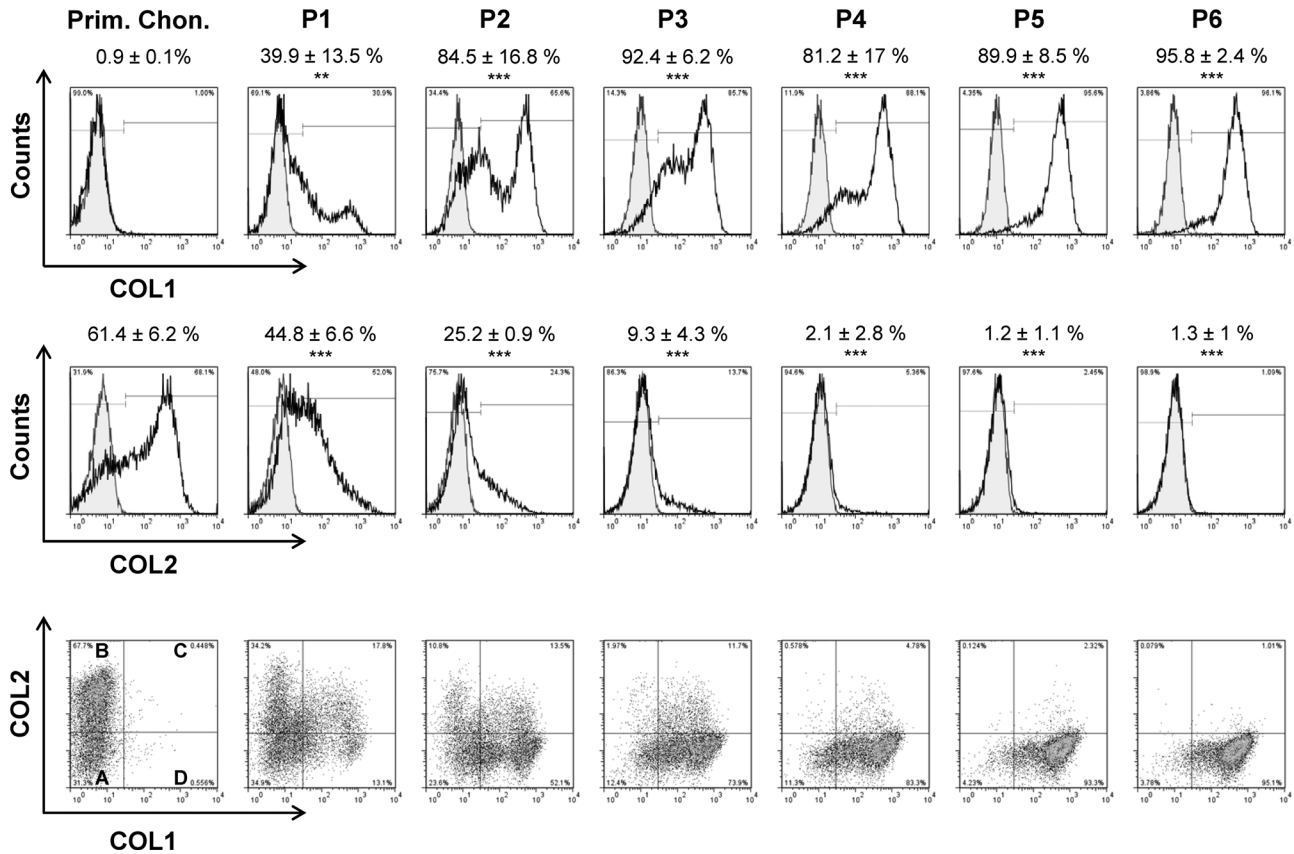

Figure 1. Intracellular collagens. Intracellular COL1 and COL2 of primary and expanded chondrocytes were assessed using flow cytometry. Values are mean ± standard deviation (SD) of the fraction of cells positive for intracellular collagens compared to the isotype control (gray) from three animals. (A) COL1 and COL2 negative, (B) COL1 negative and COL2 positive, (C) COL1 and COL2 positive, (D) COL1 positive and COL2 negative. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ as compared to primary chondrocytes.

instructions on an ABI 7500 sequence detection system (Applied Biosystems, Life Technologies, Zug, Switzerland). The following assays were used: Alpha-1 chain of COL1A1, Bt03225322_m1; COL2A1, Bt03251861_m1; Aggrecan (ACAN) Bt03212186_m1; Proteoglycan 4 (PG4; Assay-by-Design: forward primer TTATTTCTGGATGCTGACTC-CATTTA, reverse primer GGGAATGCCCCAAACTTCA, probe TCCACCACCTCCACCTCGGAGAATTA); and Versican (VCAN) Bt03217632_m1. The levels of transcripts were normalized to the expression of β2-microglobulin (B2M) Bt03251628_m1.

Flow Cytometry

To access intracellular collagens, cells were permeabilized as previously described.[12] Briefly, 2×10^5 cells in 100 μl of phosphate buffered saline (PBS; Sigma–Aldrich)/2% FBS were mixed with 1 ml cold methanol (−20°C), incubated for 15 min at 4°C, and washed with PBS/2% FBS. Cells were incubated in PBS/2% FBS instead of methanol to omit cell permeabilization. After washing, cells were incubated with anti-human COL1(2 μg/ml, clone I-8H5; Abnova, Taipei City, Taiwan) and anti-chicken COL2 (2 μg/ml, clone A2-10; Chondrex, Redmond, WA) monoclonal mouse antibodies, respectively, for 15 min at room temperature. Incubation for another 15 min with the appropriate secondary rat anti-mouse antibodies (Becton Dickinson, Basel, Switzerland) labeled with the fluorochromes APC and PE for COL2 and COL1, respectively, allowed for visualization of primary antibody binding. Isotype-matched antibodies (Becton Dickinson) were used as negative controls. Fluorescence was measured on an LSR II flow cytometry system (Becton Dickinson), and the data were analyzed using FlowJo software (www.flowjo.com).

To identify proliferating cells, primary chondrocytes were expanded for 72 h in monolayer culture and 10 μM BrdU was added at the beginning (72 h), or 16 h and 4 h before terminating the experiment. The incorporated BrdU was detected by flow cytometry according to the manufacturer's instructions (APC BrdU Flow Kit, Becton Dickinson).

Enzyme-Linked Immunosorbent Assay (ELISA)

Cells were seeded at a density of 5×10^4 cells/cm^2 in 6-well tissue culture plates and cultured for 6 days in proliferation medium with or without 50 μg/ml ascorbic acid, with the medium being changed after 3 days. To recover the secreted COL1 and COL2, the cell-associated matrix was solubilized by a sequential digestion with pepsin and elastase as described previously.[13] The COL1 and COL2 proteins in the resulting lysates were measured using collagen type I and II detection kits (Chondrex, Redmond, WA) according to the manufacturer's instructions. The levels of COL1 and COL2 proteins were normalized to the DNA content within the lysate, which was measured fluorometrically with Hoechst 33342 (Sigma–Aldrich).

Histology

Pellet cultures were fixed in 4% paraformaldehyde and embedded in paraffin for subsequent preparation of 5 μm

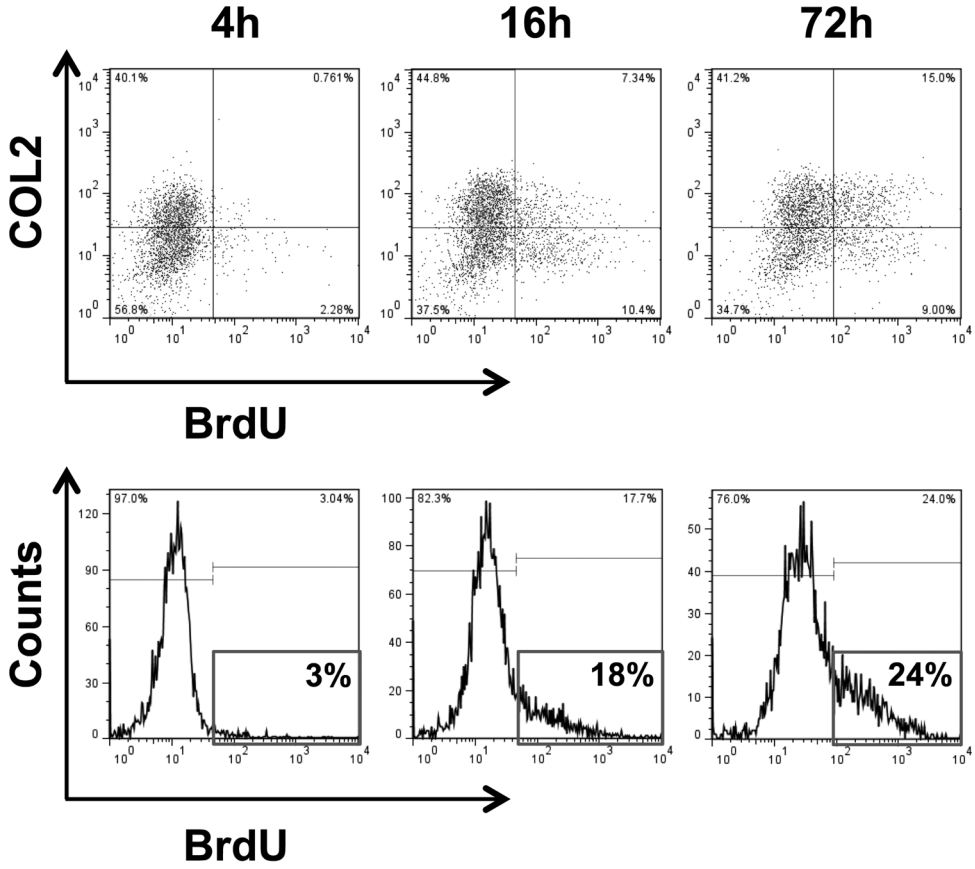

Figure 2. Proliferation. Primary chondrocytes were labeled with BrdU at different time periods before the end of the culture. Incorporated BrdU, in combination with intracellular COL2, was assessed using flow cytometry.

thick sections. Sulfated glycosaminoglycans were detected with Safranin O staining.[14] COL2 protein was detected by immunohistochemistry with an anti-chicken type II collagen monoclonal mouse antibody (0.1 μg/ml, clone A2-10, Chondrex), followed by a secondary antibody directed against mouse/rabbit antibodies conjugated to a peroxidase-labeled polymer (Envision Dual Link System-HRP, Dako, Baar Switzerland). The bound antibody was visualized using the peroxidase-specific substrate 3,3′-diaminobenzidine (Dako). The sections were counterstained with Meyer's hematoxylin.

Statistical Analysis

Differences in the levels of transcripts and proteins were evaluated by one-way ANOVA with Bonferroni's post-hoc test using GraphPad Prism version 6 for Windows. A p-value < 0.05 was considered significant.

RESULTS

Monolayer Cultures

Articular chondrocytes were expanded in monolayer cultures for up to six passages, corresponding to 21.8 ± 5.3 population doublings (PDs, Table 1).

Gene Expression

Levels of transcripts coding for COL2A1, ACAN, and PG4 decreased to <0.05%, 3%, and 0.5%, respectively, at P6 when compared to levels of primary chondrocytes (Table 1). Levels of transcripts coding for COL1A1 and VCAN increased by a factor of 205 and 4.3, respectively, at P1 when compared to those of primary chondrocytes, and these levels were maintained at later passages.

Intracellular Collagens

Intracellular levels of COL1 and COL2 proteins in permeabilized cells were assessed by flow cytometry (Fig. 1). For primary chondrocytes, $61.4 \pm 6.2\%$ of the cells stained positive for COL2. During further expansion in monolayer culture, the fraction of COL2-positive cells decreased to $1.3 \pm 1.0\%$ at P6. COL1was detected in $0.9 \pm 0.1\%$ of primary chondrocytes, and the fraction increased to >95% at P6. At all time points the population of expanded cells was heterogenous and composed of either COL1-positive (D in Fig. 1), COL2-positive (B), double positive (C), or double negative cells (A). Without cell permeabilization, no specific fluorescence signals for collagens were detected (Fig. 4, surface staining), confirming that the proteins are located intracellularly and are not bound to the cell surface.

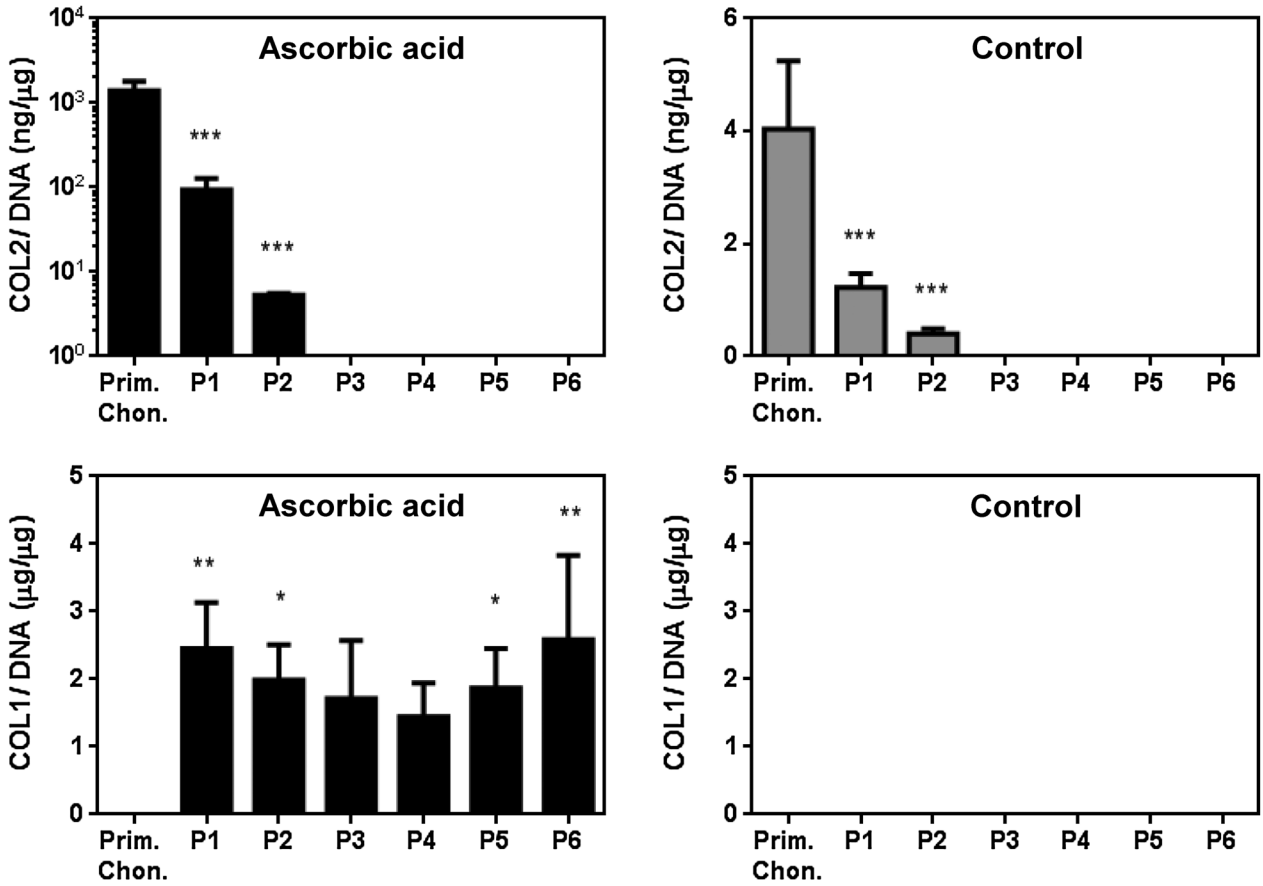

Figure 3. Collagens in the cell-associated matrix. Deposition of COL1 and COL2 into the cell-associated matrix and its dependence on ascorbic acid was assessed by ELISA. Values are mean ± standard deviation (SD) of cells from three animals. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ as compared to primary chondrocytes.

Proliferation

After 72 h of culture, 41–56% of the cells were positive for COL2 (Fig. 2). The fraction of BrdU-positive cells increased from 3% to 24% when cells were exposed to BrdU for 4–72 h. Cells that incorporated BrdU were found to be either COL2-positive or COL2-negative.

Collagens in the Cell-Associated Matrix

The amount of COL1 and COL2 proteins in the cell-associated matrix of expanded chondrocytes was assessed by ELISA (Fig. 3). In the absence of ascorbic acid, the amount of COL2 protein deposition in the matrix was 100-fold to 1,000-fold lower than cultures with 50 μg/ml ascorbic acid. In cultures with and without ascorbic acid, the amount of COL2 per DNA decreased to 0.1% and 5%, respectively, at P2 compared to that in primary chondrocytes. COL1 protein was not detectable in the absence of ascorbic acid (to note: the sensitivity of the COL2 assay is 50 times that of the COL1 assay), but in its presence, COL1 was found at P1 and later passages at similar levels.

Effect of Ascorbic Acid on Chondrocytes in Monolayer Culture

Cells were cultured in monolayer for 6 days with or without 50 μg/ml ascorbic acid. In its presence, the cell number increased by 30–50% at the end of the culture period (data not shown). The amount of intracellular COL1 and COL2 protein, estimated by the fluorescence values for the respective collagen of cells considered positive, were similar regardless whether the cells were exposed to ascorbic acid (Fig. 4). However, the fraction of COL2-positive cells was lower at P1–P3, and the fraction of COL1-positive cells was higher at P1 in the presence when compared to cultures without ascorbic acid.

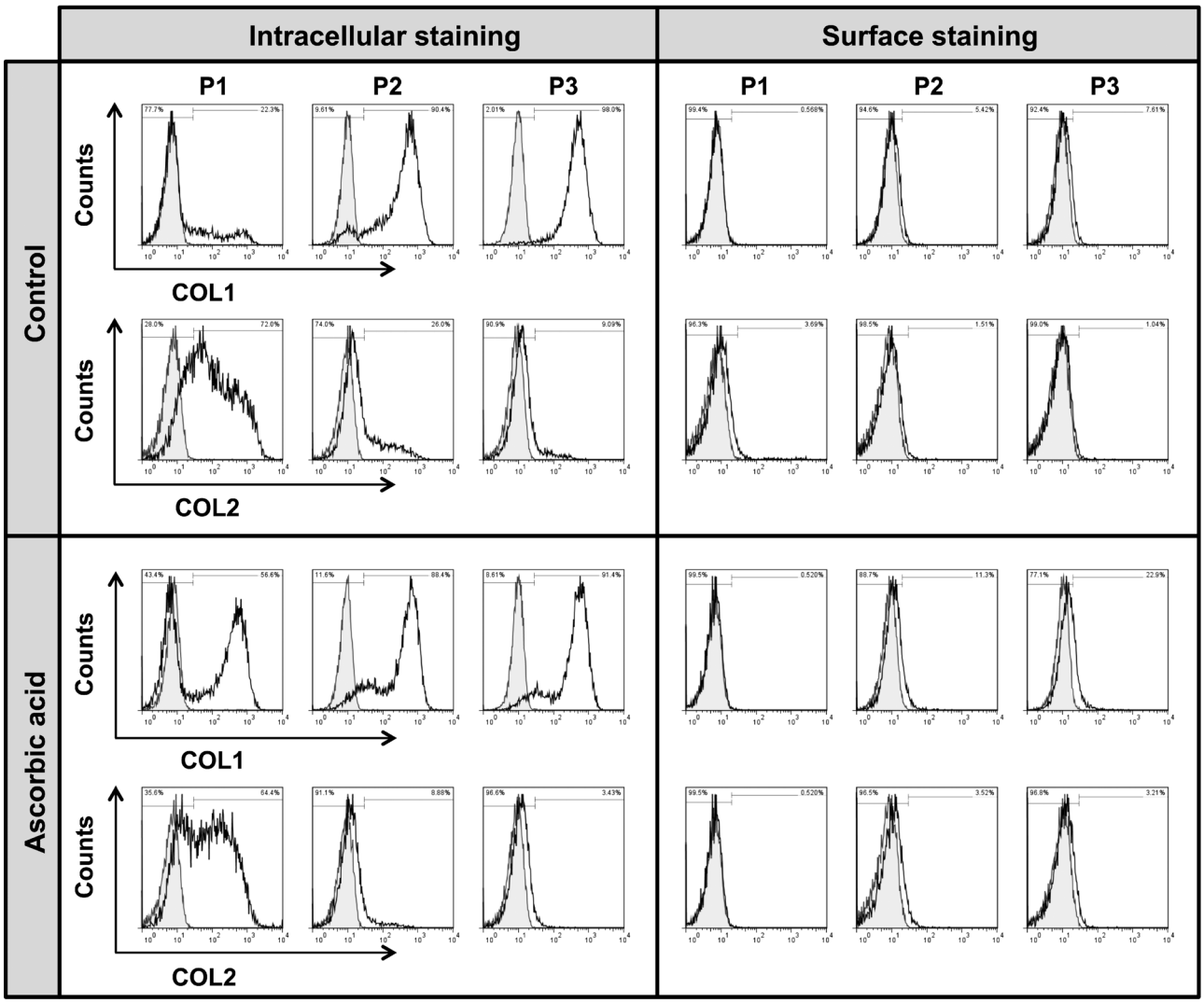

Figure 4. Intracellular collagens and ascorbic acid. Primary chondrocytes were expanded with or without (control) ascorbic acid for six days, and intracellular COL1 and COL2 (intracellular staining) was assessed using flow cytometry. Surface-bound collagen (surface staining) was assessed by omitting the cell permeabilization step during the staining procedure. Isotype controls are shown in gray.

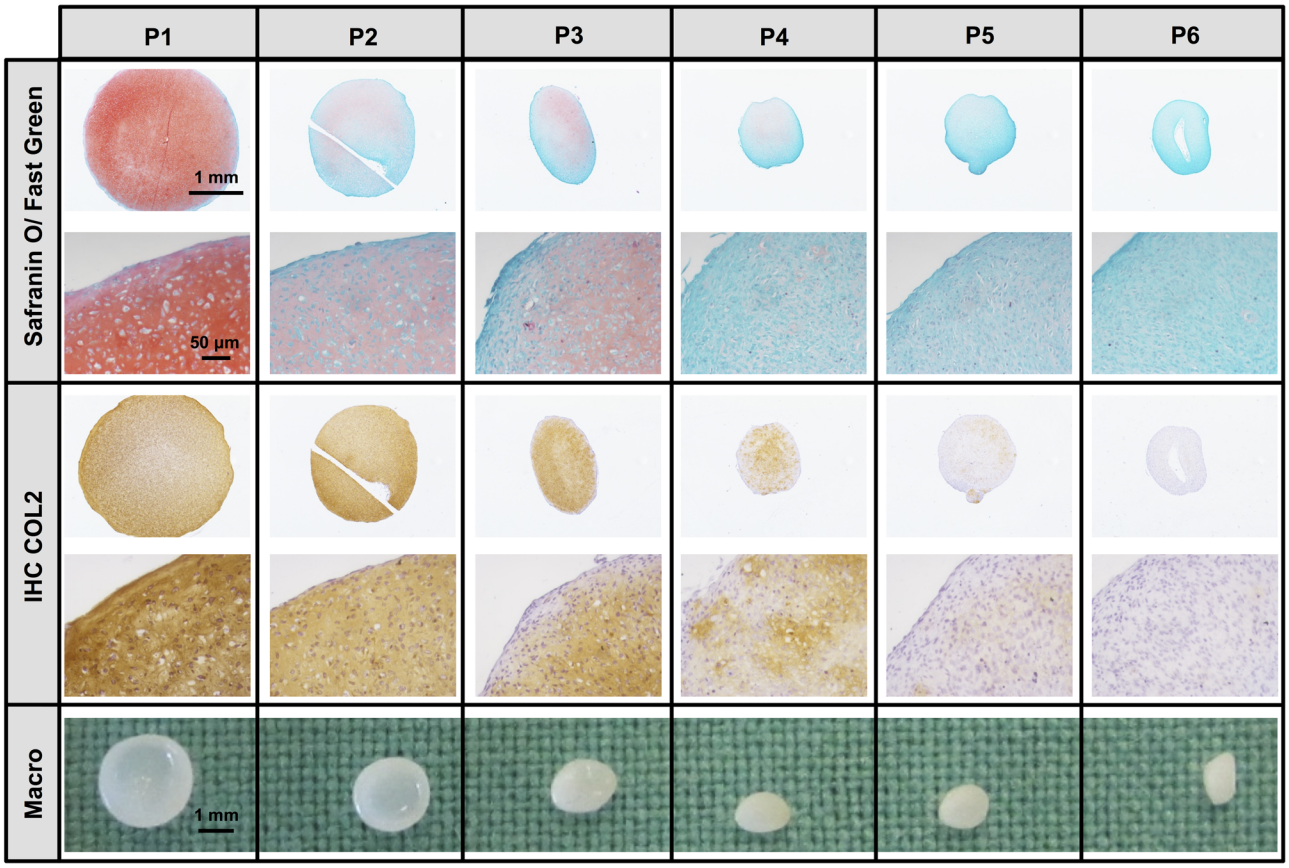

Figure 5. Pellet cultures—histological analysis. Expanded chondrocytes from P1–P6 were subjected to pellet cultures and analyzed histologically for the presence of glycosaminoglycans (Safranin-O, red staining) and collagen type II (COL2) protein (immunohistochemistry, brown staining).

Pellet Cultures

After each passage, expanded chondrocytes were subjected to pellet cultures to assess their potential to form cartilage-like tissue. Pellet cultures grown from P1 cells resulted in a flat, disc-like tissue that stained positive for glycosaminoglycans and COL2, with cells located within lacunae (Fig. 5). As the number of cell passages increased, the formed pellets acquired a spherical shape, and the intensity of glycosaminoglycan and COL2 staining decreased until pellets were negative at P6. The levels of transcripts coding for COL2A1, ACAN, and PG4 reached similar levels in pellet cultures grown from P1 cells, as observed in primary chondrocytes (Fig. 6 and Table 1). With increasing passage numbers, the levels of transcripts coding for COL2A1 and ACAN decreased to 3% and 9%, respectively, at P6 compared to P1. Levels of transcripts coding for COL1A1, VCAN, and PG4 were found to fluctuate with a maximum fivefold difference between the pellet cultures.

DISCUSSION

The purpose of this study was to elucidate the phenotypic adaptations that articular chondrocytes undergo in subsequent monolayer culture after harvesting from cartilage tissue, with particular focus on the expression of the COL2 and COL1 proteins.

In monolayer culture, adaptation of the chondrocytes isolated from the cartilage tissue to the new environment was marked by the initiation of cell proliferation, a decrease in the levels of the chondrocyte-specific transcripts encoding COL2, ACAN, and PG4, and an increase in the levels of transcripts encoding COL1 and VCAN, which has also been observed by others.[4,7] Similar to the levels of transcripts encoding COL2, the fraction of COL2-positive cells detected by flow cytometry and the deposition of COL2 in the cell-associated matrix decreased with the increase in passage number. While transcripts encoding COL2 remained detectable after P2, the intracellular and secreted proteins were not measurable after this passage. This apparent discrepancy is likely due to the different sensitivities of the two technological approaches used, with real-time PCR presenting a much higher sensitivity than the antigen-detection-based methods of flow cytometry and ELISA. However, it must also be considered that post-transcriptional regulation, for example, by micro RNAs[15,16] may lead to the inhibition of COL2 translation while the respective transcripts are still present.

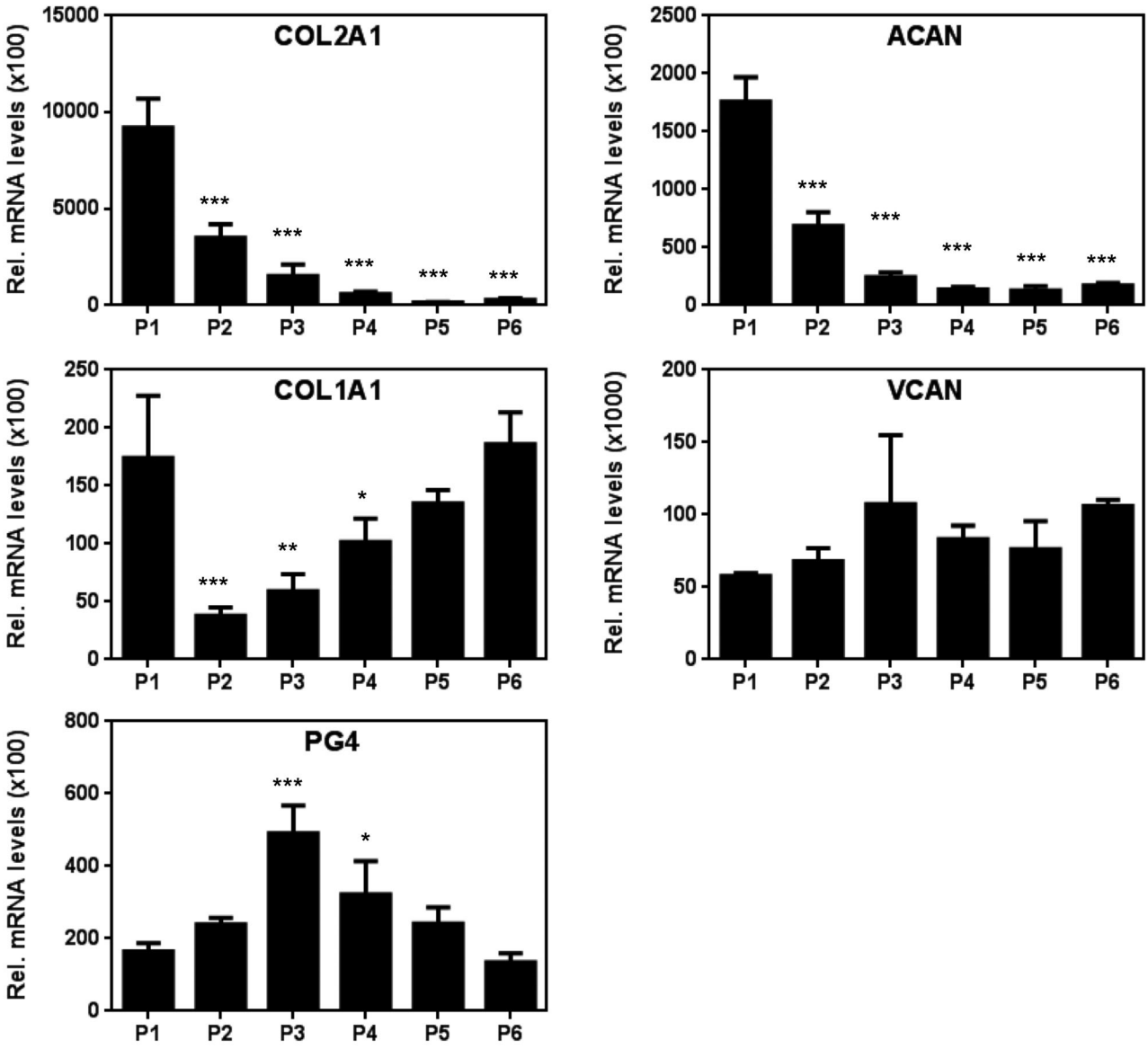

Figure 6. Pellet cultures—gene expression. Expanded chondrocytes from P1–P6 were subjected to pellet cultures, and the levels of transcripts coding for COL2A1, ACAN, COL1A1, VCAN, and PG4 were measured. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ as compared to P1.

Applying flow cytometry to detect intracellular COL1 and COL2 proteins concomitantly allowed us to investigate the different phenotypes present within a pool of expanded cells, an analysis which is forfeit when measuring the levels of transcripts alone. In primary chondrocytes, approximately 70% of the cells stained positive for COL2. During expansion, COL1 and COL2 are not solely synthesized; rather, a considerable fraction of cells simultaneously express both types of collagen. There are two paths the COL2-positive cells may follow during expansion: (i) cells stop synthesizing COL2 and then start to produce COL1, or, more likely; (ii) the cells switch from a COL2-positive to a mixed COL2/COL1-positive phenotype, and end in a COL1-positive phenotype. In support of our data is a recent study by Wu et al.,[17] where expanded chondrocytes were allowed to form a pericellular matrix in 3D culture, and it was determined that the fraction of cells able to produce a COL2-positive pericellular matrix decreased with increasing time in monolayer culture. Other studies by Shahdadfar et al.[18] and Marlovits et al.[5] have reported that with increasing cell expansion time, the levels of intracellular COL2 protein decreased, whereas COL1 levels increased. However, there was no concomitant detection of the two types of collagen, and no heterogeneous populations with respect to the expression of these proteins were reported.

When the resting primary articular chondrocytes were subjected to monolayer culture, they adhered and began to proliferate within a few days. As approximately 70% of the primary chondrocytes expressed COL2, we wondered whether the proliferating pool of cells was comprised of COL2-positive cells or whether the pool

was restricted to COL2-negative cells. The data presented confirm that the proliferating cells presented both phenotypes, COL2-positive and COL2-negative. This is of particular interest since these experiments showed that cells harvested from articular cartilage tissue are able to maintain synthesis of COL2 while proliferating in monolayer culture. This is encouraging for strategies seeking to maintain COL2 synthesis while increasing cell numbers for cartilage repair therapies.

To detect the cell matrix-associated collagens, ascorbic acid was added to the monolayer cultures. Ascorbic acid is present in media formulations used for pellet cultures, but only occasionally in formulations used to expand articular chondrocytes in monolayer cultures. To test whether the intracellular pools of the collagens were affected, chondrocytes were expanded for three passages either with or without ascorbic acid. The strength of the fluorescence signal, which is a measure of the amount of intracellular proteins, of COL1- and COL2-positive cells did not change upon addition of ascorbic acid. Thus, similar amounts of intracellular collagen were maintained, implying that no negative feedback mechanisms or overloading of cells due to incomplete post-translational modifications (lack of proline hydroxylation) occurred. However, the fraction of cells positive for COL2 was lower in the presence of ascorbic acid, whereas the fraction of COL1 was higher. In addition, we found more cells at the end of the experiments when ascorbic acid was added to the cultures, thus the cells went through more PDs, which is in agreement with previous publications.[19,20] The higher number of PDs may account for the differences in the fraction of COL1- and COL2-positive cells, as a similar observation was made in the present study, although in the absence of ascorbic acid, when the cells were expanded over several passages (Fig. 1).

The potential of expanded chondrocytes to form cartilage-like tissue in the absence of chondrogenic growth factors was tested with pellet cultures. We found that with increasing numbers of PDs, the cells' potential to form cartilage-like tissue was diminished, as evidenced by decreased levels of transcripts encoding cartilage specific proteins and decreased staining intensities for deposited glycosaminoglycans and COL2, which is consistent with previous observations.[7,21] Regardless of the number of PDs at which the cells were collected, COL1 was found in the respective pellet cultures. Importantly, the decreased expression of intracellular COL2 and its incorporation in the extracellular matrix during monolayer cultures was paralleled by the cells' decreased potential to form cartilage-like tissue. It was previously shown that co-cultures of freshly prepared and expanded chondrocytes still resulted in cartilage tissue formation when the fraction of fresh chondrocytes was as low as 5–20%.[22] Interestingly, this matches the fraction of COL2-positive cells that was detected around P3/P4 in the present study, after which the potential for formation of cartilage-like tissue is lost. Moreover, Wu et al.[17] recently showed that the ability to form cartilage tissue in the presence of chondrogenic transforming growth factor beta 3 was substantially higher in cells that were able to produce a COL2-positive pericellular matrix. This leads one to speculate that the COL2 secreted by cells in the early phases of the pellet cultures is actively contributing to the microenvironment by acting in a chondroinstructive manner on neighboring cells, similar to the chondroinstructive properties of COL2 matrices.[9,10] However, it remains to be determined whether cell-derived COL2 is functionally critically involved in the formation of cartilage-like tissue or whether it can only be considered as a surrogate marker.

If it holds true that the fraction of COL2-positive cells within a mixed chondrocyte lineage population (in the current study 5–20%) is key to inducing the formation of cartilage-like tissue, this approach of intracellular staining for COL2 may be used to predict the effectiveness of expanded chondrocytes to form cartilage-like tissue.

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