Journal of Cellular Biochemistry 98:1140–1149 (2006)

Expansion on Specific Substrates Regulates the Phenotype and Differentiation Capacity of Human Articular Chondrocytes

Andrea Barbero,[1] Shawn Patrick Grogan,[2] Pierre Mainil-Varlet,[2] and Ivan Martin[1]*

[1]Departments of Surgery and of Research, University Hospital, Basel, Switzerland
[2]Osteoarticular Research Group, Institute of Pathology, University of Bern, Switzerland

Abstract In this study, we investigated if monolayer expansion of adult human articular chondrocytes (AHAC) on specific substrates regulates cell phenotype and post-expansion multilineage differentiation ability. AHAC isolated from cartilage biopsies of five donors were expanded on plastic dishes (PL), on dishes coated with collagen type II (COL), or on slides coated with a ceramic material (Osteologic™, OS). The phenotype of expanded chondrocytes was assessed by flow cytometry and real-time RT-PCR. Cells were then cultured in previously established conditions promoting differentiation toward the chondrogenic or osteogenic lineage. AHAC differentiation was assessed histologically, biochemically, and by real-time RT-PCR. As compared to PL-expanded AHAC, those expanded on COL did not exhibit major phenotypic changes, whereas OS-expanded cells expressed (i) higher bone sialoprotein (BSP) (22.6-fold) and lower collagen type II (9.3-fold) mRNA levels, and (ii) lower CD26, CD90 and CD140 surface protein levels (1.4–11.1-fold). Following chondrogenic differentiation, COL-expanded AHAC expressed higher mRNA levels of collagen type II (2.3-fold) and formed tissues with higher glycosaminoglycan (GAG) contents (1.7-fold), whereas OS-expanded cells expressed 16.5-fold lower collagen type II and generated pellets with 2.0-fold lower GAG contents. Following osteogenic differentiation, OS-expanded cells expressed higher levels of BSP (3.9-fold) and collagen type I (2.8-fold) mRNA. In summary, AHAC expansion on COL or OS modulated the de-differentiated cell phenotype and improved the cell differentiation capacity respectively toward the chondrogenic or osteogenic lineage. Phenotypic changes induced by AHAC expansion on specific substrates may mimic pathophysiological events occurring at different stages of osteoarthritis and may be relevant for the engineering of osteochondral tissues. J. Cell. Biochem. 98: 1140–1149, 2006.

Key words: chondrocyte; cartilage; chondrogenic differentiation; multilineage differentiation; osteoarthritis

Adult human articular chondrocytes (AHAC) during monolayer expansion undergo a process of de-differentiation, acquiring a fibroblast-like phenotype [Benya and Shaffer, 1982; Binette et al., 1998]. In this de-differentiated state, chondrocytes exhibit a certain degree of plasticity, being able to differentiate toward various mesenchymal phenotypes (i.e., chondrogenic, osteogenic, and adipogenic), in response to specific microenvironmental factors [Barbero et al., 2003; Dell'Accio et al., 2003; Tallheden et al., 2003]. The post-expansion multilineage differentiation ability of AHAC has been shown to be modulated by the use of specific growth factors during the expansion phase [Jakob et al., 2001; Barbero et al., 2003]. The concept that the conditions of AHAC expansion may regulate their subsequent differentiation capacity may well be extended toward other culture parameters, beyond the standard application of soluble factors. For instance, since the biology of articular chondrocytes is highly influenced by their interaction with specific extracellular matrix (ECM) molecules [Scully et al., 2001; van der Kraan et al., 2002], the composition of the substrate used for AHAC expansion could also modulate the cell post-expansion differentiation ability.

So far, the effects of chondrocyte expansion on different substrates have been mostly investigated only in terms of phenotypic changes,

Grant sponsor: Swiss National Science Foundation; Grant number: 4046-58623.

*Correspondence to: Ivan Martin, Institute for Surgical Research, University Hospital Basel, Hebelstrasse 20, ZLF, Room 405, 4031 Basel, Switzerland.
E-mail: imartin@uhbs.ch

Received 20 September 2005; Accepted 16 November 2005
DOI 10.1002/jcb.20754

using cells from different animal species and with sometimes controversial findings. Shakibaei et al. [1997] showed that chicken epiphyseal chondrocytes cultured on type II collagen remained rounded for a longer time (up to 17 days) than cells expanded on plastic, suggesting a better maintenance of the differentiated phenotype. Similar results were obtained more recently by Kino-Oka et al. [2005] using rabbit articular chondrocytes expanded on a type I collagen substrate. In contrast, Brodkin et al. [2004] reported that bovine articular chondrocyte expansion on substrates coated with collagen type II, collagen type I or fibronectin did not alter cell morphology or expression of genes typically used to characterize the stage of chondrocyte differentiation.

In the present work, we investigated whether expansion of human articular chondrocytes on various ECM molecules would not only differentially influence the stage of cell differentiation, but also modulate their multilineage differentiation ability. In particular, we hypothesized that a substrate coated with collagen type II, a specific component of articular cartilage matrix, will improve the capacity of AHAC to re-differentiate toward the chondrogenic lineage, while a substrate coated with a ceramic-based material, mimicking the inorganic phase of bone tissue, will increase their osteogenic differentiation ability. Fibronectin, which is abundant in a wide variety of mesenchymal tissues, was used in this study as a control coating. We then aimed at determining whether the possible cellular responses induced by AHAC expansion on the different substrates could be associated to changes in the expression of specific surface molecules, recently described for the characterization of AHAC phenotype [Diaz-Romero et al., 2005].

MATERIALS AND METHODS

Substrate Preparation

Collagen type II (COL) and fibronectin (FN) substrates were prepared by coating tissue culture plastic dishes (PL) respectively with 1 μg/ml bovine collagen type II (Sigma-Aldrich) or 1 μg/ml human fibronectin (Sigma-Aldrich). After overnight incubation, dishes were washed twice with phosphate buffered saline (PBS) before use. The bioceramic substrate used in this study (BioCoattrade™ Osteologic™ by Millenium Biologix, OS) was a mixture of calcium hydroxyapatite and a silicon stabilized tricalcium phosphate, deposited on glass slides as previously described [Langstaff et al., 1999]. PL and OS substrates were also washed twice in PBS prior to use.

Cell Isolation

Human articular cartilage samples were collected post mortem (within 24 h after death) after informed consent and in accordance with the local Ethical Commission from the knee joints of five individuals (mean age 49.6 years, range 18–70), with no history and no radiographic signs of joint disease. Chondrocytes were isolated using 0.15% type II collagenase for 22 h 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 (complete medium).

Cell Adhesion and Expansion

AHAC were plated on PL, COL, FN, or OS at 10^4 cells/cm^2 in complete medium with the addition of 1 ng/ml of transforming growth factor-β1, 5 ng/ml of fibroblast growth factor-2 and 10 ng/ml of platelet-derived growth factor-bb and cultured in a humidified 37°C/5% CO_2 incubator. This medium was previously shown to enhance AHAC proliferation rate and responsiveness to chondrogenic and osteogenic stimuli [Barbero et al., 2003]. After 4 h of incubation, cells that did not adhere to the substrates were counted using trypan blue. The adherent AHAC were then expanded for two passages as previously described [Barbero et al., 2003] and either assessed for gene expression and flow cytometry, or induced to differentiate toward the chondrogenic and osteogenic lineages.

Cell Differentiation Assays

Chondrogenic differentiation. The chondrogenic capacity of post-expanded AHAC was investigated in pellet cultures using a defined serum-free medium, as previously described [Barbero et al., 2003]. Briefly, AHAC were suspended in DMEM supplemented with ITS^{+1} (Sigma Chemical, St. Louis, MO), 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 (chondrogenic medium). Aliquots of 5×10^5 cells/0.5 ml were

centrifuged at 250*g* for 5 min in 1.5 ml polypropylene conical tubes (Sarstedt, Nümbrecht, Germany) to form spherical pellets, which were placed onto a 3D orbital shaker (Bioblock Scientific, Frenkendorf, Switzerland) at 30 rpm. Pellets were cultured for 2 weeks, with medium changes twice per week, and subsequently processed for histological, immunohistochemical, biochemical, or mRNA analysis as described below. Each analysis was performed independently in at least two entire pellets for each primary culture and expansion condition.

Osteogenic differentiation. The osteogenic differentiation capacity of post-expanded AHAC was investigated in monolayer culture in the presence of osteogenic supplements, as previously described [Barbero et al., 2003]. Briefly, AHAC were seeded at 5×10^3 cells/cm^2 in complete medium supplemented with 10 mM β-glycerophosphate, 10 nM dexamethasone, and 0.1 mM L-ascorbic acid-2-phosphate (osteogenic medium) and cultured for 3 weeks, with medium changes twice a week. Cell layers were subsequently processed by histochemical or mRNA analysis as described below. Each analysis was performed independently in at least two cell layers for each primary culture and expansion condition.

Analytical Assays

Real-time quantitative RT-PCR assays. RNA was extracted using Trizol (Life Technologies, Basel, Switzerland), according to the manufacturer's protocol. Post-expanded cells, pellets cultured in chondrogenic medium and cell layers cultured in osteogenic medium were first sonicated for 1 min while in Trizol. RNA was treated with DNAseI using the DNA-free™ Kit (Ambion) and quantified spectrofluorimetrically. cDNA was generated from 3 μg of RNA by using 500 μg/ml random hexamers (Catalys AG, CH) and 1 μl of 50 U/ml Stratascript™ reverse transcriptase (Stratagene, NL), in the presence of dNTPs.

PCR reactions were performed and monitored using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer/Applied Biosystems, Rotkreuz, Switzerland). Cycle temperatures and times as well as primers and probes used for the reference gene (18-S rRNA) and the genes of interest (collagen type I, collagen type II, collagen type X, bone sialoprotein-2, BSP, and osteocalcin, OC), were as previously described [Barbero et al., 2003]. Collagen types I and II were used to define the stage of chondrogenic differentiation, BSP and OC were used as markers for osteogenic differentiation, and collagen type X as a marker for hypertrophic differentiation. Each sample was assessed at least in duplicate for each gene of interest.

Flow cytometric analysis of expanded AHAC. Cell preparation, staining and flow cytometry was conducted as previously described [Diaz-Romero et al., 2005]. Briefly, cells were rinsed in PBS and resuspended at 5×10^6 cells/ml. A three-color immunofluorescence analysis for different surface markers was performed by simultaneous labeling with mAb-fluorescein biothiocynate (FITC), mAb-Phycoerythrin (PE), and 7-amino-actinomycin D (7-AAD). Non-specific staining was assessed using fluorochrome- and isotype-matched immunoglobulins (isotype control). Details of the monoclonal antibodies and of the flow cytometer used in this study can be found in a previous report [Diaz-Romero et al., 2005]. For each sample, a region for live cells (cells excluding 7-AAD) was defined and at least 1×10^4 live chondrocytes were analyzed for each labeling. Data analysis and mean fluorescence intensity (MFI) were determined using FlowJo software (version 3.4, Tree Star, Inc., San Carlos, CA). The level of marker expression was calculated as the ratio between MFI of sample cells and that of the isotype control. To establish differences in surface marker expression, the MFI values of AHAC expanded on COL, FN, and the OS were expressed as fold difference of values measured in PL-expanded cells.

Histological, histochemical, and immunohistochemical analyses of differentiated AHAC. Cell pellets cultured in chondrogenic medium were fixed in 4% formalin, embedded in paraffin, cross-sectioned (5 μm thick), and stained with Safranin O for sulfated glycosaminoglycans (GAG). Sections were also processed for immunohistochemistry using antibodies against collagen type I (Quartett, Berlin, Germany), type II (II-II6B3, Hybridoma Bank, University of Iowa, USA), or type X (Quartett), as previously described [Grogan et al., 2003]. Cell layers cultured in osteogenic medium were stained for alkaline phosphatase (AP) using Sigma Kit 86-C, according to the manufacturer's instructions.

Biochemical analyses of differentiated AHAC. Cell pellets cultured in chondrogenic medium 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 amounts were measured spectrophotometrically using dimethylmethylene blue [Farndale et al., 1986], with chondroitin sulfate as a standard, and normalized to the DNA amounts, measured spectrofluorometrically using the CyQUANT® Kit (Molecular Probes, Eugene), with calf thymus DNA as a standard.

Cell layers cultured in osteogenic medium were rinsed in PBS and scraped in 0.01% sodium dodecyl sulfate. AP activity levels were measured in triplicate aliquots as the rate of conversion of *p*-nitrophenyl phosphate using Sigma Kit 104. AP activity was expressed as nM of transformed *p*-nitrophenol (pNP) per min and per μg of DNA.

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

RESULTS

Chondrocyte Adhesion and Proliferation Ability on the Different Substrates

AHAC were seeded on the different substrates and incubated for 4 h. After this time, the percentage of adherent cell differed largely between each primary culture (range 70%–95%), but was similar (less than 10% variation) using the different substrates. Adherent AHAC from different primary cultures proliferated at comparable rates (average of 8.8 ± 1.2 doublings during 14 ± 3 days), which were similar (less than 5% variation in the number of doublings/day) using different substrates.

Gene Expression Profiles of Chondrocytes Expanded on the Different Substrates

After expansion on the different substrates, the stage of AHAC differentiation was assessed by measuring the mRNA expression levels of genes encoding for collagen type II, collagen type I and bone sialoprotein (BSP) (Fig. 1A). As compared to chondrocytes cultured on PL, those cultured on COL expressed significantly less BSP (2.1-fold), while those expanded in OS expressed significantly more BSP (22.6-fold) and less collagen type II (9.3-fold). None of the genes analyzed was differentially modulated by AHAC culture on FN.

Flow Cytometric Analysis

Flow cytometric analysis of expanded AHAC indicated that the selected substrates differentially modulated the expression of several surface molecules, with the largest differences observed following expansion on OS (Fig. 1B). As compared to AHAC expanded on PL, those expanded on OS expressed lower levels of CD26 (peptidase IV, 3.3-fold), CD90 (Thy-1, 1.4-fold), and CD140a (PDGF receptor α, 11.1-fold); COL-expanded AHAC expressed higher levels of CD44 (hyaluronan receptor, 1.2-fold) and CD105 (endoglin, 1.1-fold), whereas FN-expanded AHAC expressed higher levels of CD105 (1.2-fold) and lower levels of CD90 (Thy-1, 1.2-fold).

The surface molecules CD14 (LPS receptor), CD49a (α1 integrin chain), CD51/61(αVβ3 integrin), CD54 (ICAM-1), CD95 (FAS), CD106 (VCAM-1), CD119 (γ-interferon receptor), CD120a (TNF-receptor I), and CD221 (IGF-1 receptor alpha) were either not detected or expressed at very low levels (less than two-fold difference from the isotype control) by AHAC expanded on the investigated substrates.

Chondrogenic Differentiation

After 2 weeks of culture in chondrogenic medium as pellets, AHAC expanded on PL, COL, or FN were able to generate hyaline-like cartilaginous tissues intensely stained for GAG and collagen type II and faintly stained for collagen type I (Fig. 2A). The staining intensity for GAG and collagen type II appeared to be relatively more intense in pellets generated by COL-expanded cells. In contrast, chondrocytes expanded on OS formed pellets faintly stained for GAG and collagen type II and strongly stained for collagen type I (Fig. 2A). Collagen type X stain was at background levels in all the pellets (data not shown).

Biochemical analysis quantitatively confirmed that pellets generated by COL-expanded AHAC contained significantly higher (1.7-fold) GAG/DNA contents than those generated by PL-expanded cells (Fig. 2B). Instead, pellets generated by OS-expanded chondrocytes contained

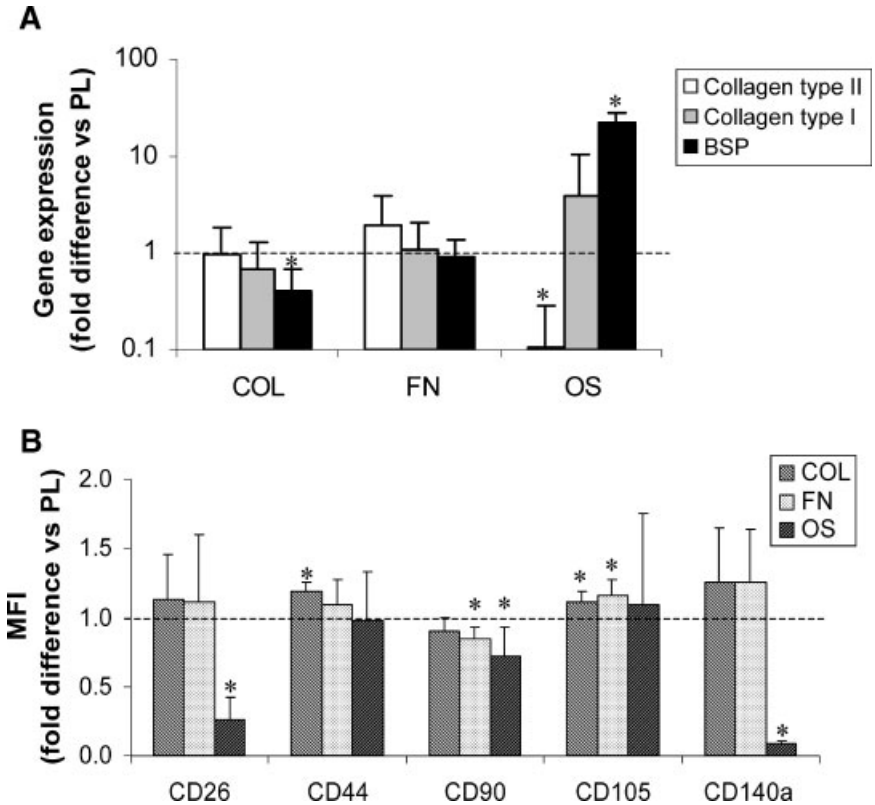

Fig. 1. Phenotypic characterization of adult human articular chondrocytes (AHAC) cultured on the different substrates. AHAC isolated from cartilage biopsies of five different donors were expanded on standard plastic dishes (PL), on dishes coated with collagen type II (COL), on dishes coated with fibronectin (FN) or on glasses coated with a synthetic bioactive bone-like ceramic (BioCoat™ Osteologic™ by Millenium Biologix, OS). **A**: Real time RT-PCR analysis of the mRNA expression of collagen types II, collagen type I and bone sialoprotein (BSP) in the post expanded cells. Levels are expressed as fold of difference from those measured in PL-expanded AHAC and reported as mean ± SD. **B**: Flow cytometric analysis of post-expanded cells; the markers reported are those whose expression was significantly modulated by any substrate. Mean fluorescence intensity (MFI) of the CD markers are expressed as fold of difference from the level measured in PL-expanded AHAC and reported as mean ± SD. (*$P < 0.05$ from expansion on PL).

markedly and significantly lower (2.0-fold) GAG/DNA amounts.

As compared to cells expanded on PL, collagen type II mRNA of COL- and OS-expanded chondrocytes was respectively 2.3-fold higher and 16.5-fold lower after chondrogenic differentiation in pellets; conversely, collagen type I expression was 2.7-fold lower in COL- and 3.5-fold higher in OS-expanded chondrocytes (Fig. 2C). Collagen type I and II expression was similar in pellets generated by PL- and FN-expanded cells. Collagen type II mRNA expression levels in pellets generated by COL-expanded AHAC were 4.4-fold lower than those measured in the corresponding native cartilage tissues. The expression of BSP was not detectable in pellets from any group. Collagen type X expression was close to the limit of detection and similar in pellets from all groups (data not shown).

Osteogenic Differentiation

After 3 weeks of culture in osteogenic medium, AHAC expanded on all the substrates were in large numbers positively stained for AP (Fig. 3A). While cells expanded on PL, COL, and FN were generally fibroblastic in shape, OS-expanded AHAC exhibited a cuboidal morphology, resembling that of osteoblast-like cells. AP activity quantified by enzymatic assays was similar in cells expanded in the different substrates (less than 5% variation between groups). As compared to PL-expanded chondrocytes, only OS-expanded AHAC significantly increased the mRNA expression of the osteoblast-related genes collagen type I (3.9-fold) and BSP (2.8-fold) after osteogenic induction, while OC mRNA expression was similar in all cultures (Fig. 3B). The mRNA expression levels of collagen type I, BSP and OC by OS-expanded

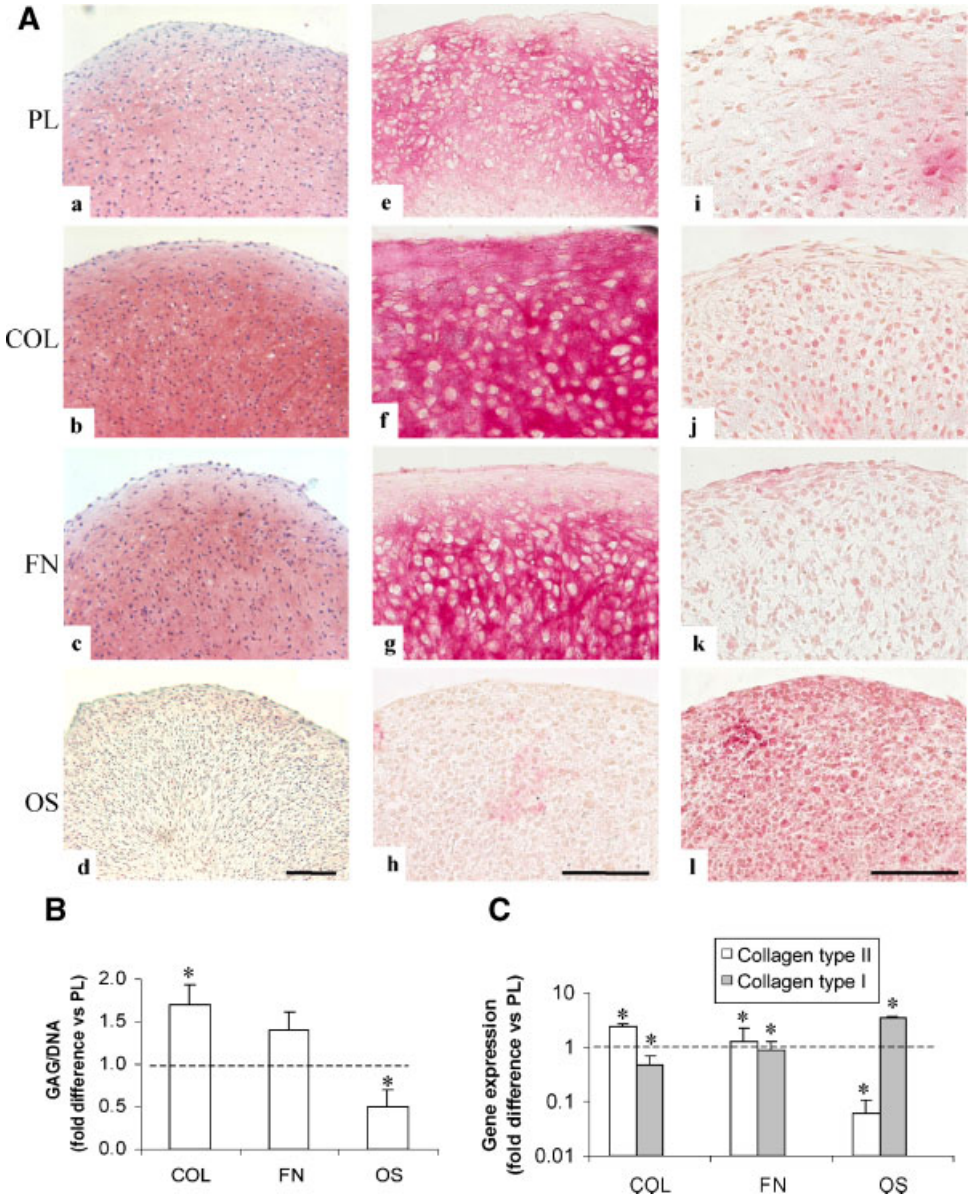

Fig. 2. Chondrogenic differentiation of adult human articular chondrocytes (AHAC) cultured on the different substrates. **A**: Safranin O (**a**–**d**), collagen type II immunohistochemical (**e**–**h**) and collagen type I immunohistochemical (**i**–**l**) stainings of representative pellets generated by chondrocytes expanded on PL (**a**, **e** and **i**), COL (**b**, **f** and **j**), FN (**c**, **g** and **k**), or OS (**d**, **h** and **l**), after 2 weeks' culture in chondrogenic medium. Bar = 100 μm. **B**: Sulfated glycosaminoglycan (GAG) content of the pellets, reported as μg GAG/μg DNA. Levels are expressed as fold of difference from those measured in pellets generated by PL-expanded AHAC and reported as mean ± SD. **C**: Real time RT-PCR analysis of the mRNA expression of collagen types II and I by AHAC after pellet culture. Levels are expressed as fold of difference from those measured in pellets generated by PL-expanded cell and reported as mean ± SD. mRNA expression of BSP was not detectable in any experimental group. (*statistically significant difference from pellets generated by PL-expanded cells). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cells after osteogenic differentiation were respectively 9.0-fold higher, 24.7-fold lower, and 4.3-fold lower than those previously measured in human osteoblast cultures [Frank et al., 2002]. Collagen type II mRNA was expressed at basal levels in all experimental groups, with no statistically significant differences between cultures established using cells expanded in the different substrates. Collagen type X expression was close to the level of

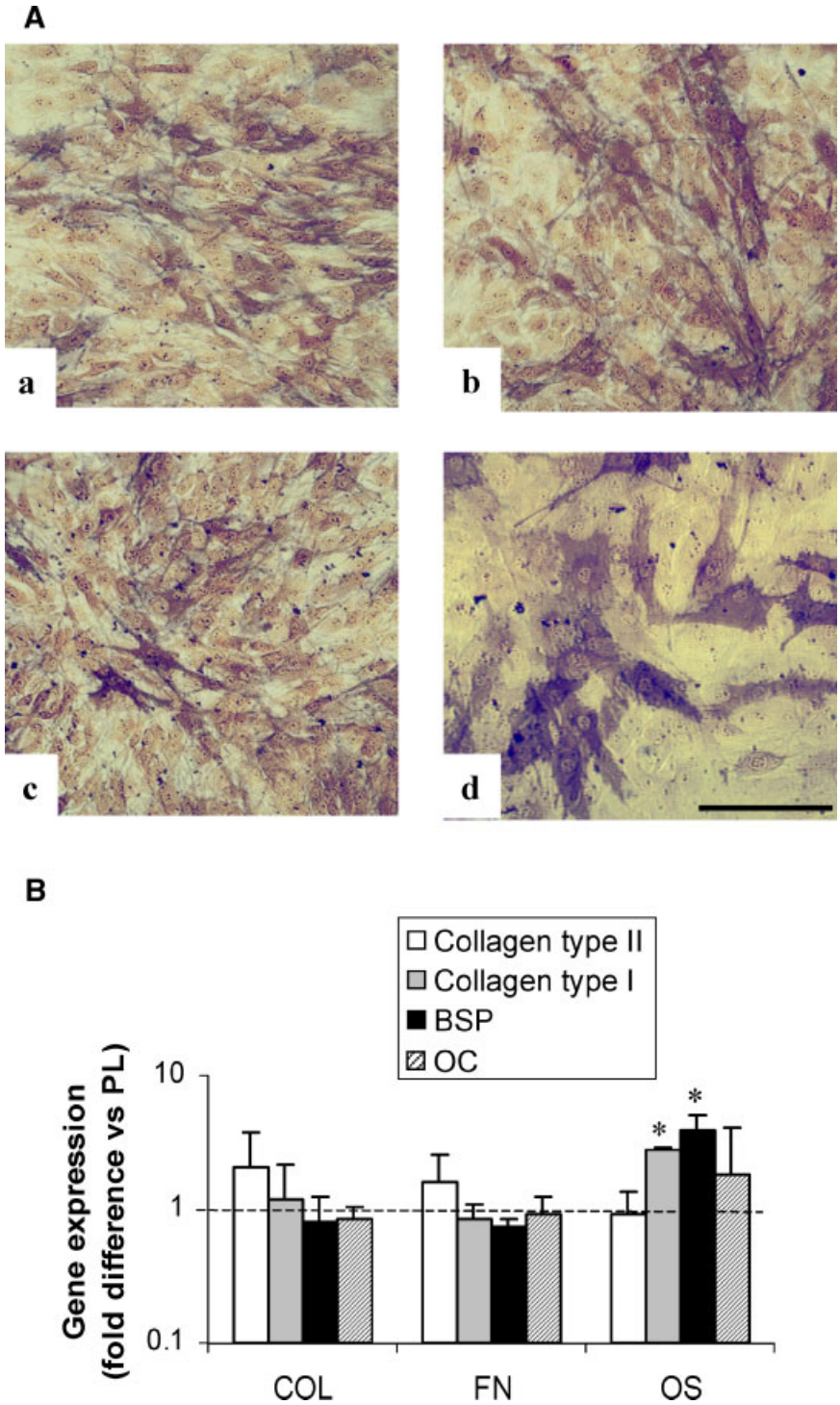

Fig. 3. Osteogenic differentiation of adult human articular chondrocytes (AHAC) cultured on the different substrates. **A**: Alkaline Phosphatase staining of representative layers of PL- (**a**), COL- (**b**), FN- (**c**), and OS- (**d**) expanded AHAC, after 3 weeks' culture in osteogenic medium. Bar = 200 μm. **B**: Real-time RT-PCR analysis of the mRNA expression of collagen type II, collagen type I, bone sialoprotein (BSP) and osteocalcin (OC) by AHAC after osteogenic differentiation. (*statistically significant difference from layers generated by PL-expanded cells). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

detection on PL-expanded AHAC and it was highly variable on COL-, FN-, or OS-expanded cells, resulting in no statistically significant difference between experimental groups.

DISCUSSION

In this study, we have demonstrated that monolayer expansion of adult human articular chondrocytes (AHAC) on different substrates regulates cell phenotype and post-expansion differentiation ability. In particular, we found that a substrate coated with collagen type II (COL) increased the capacity of AHAC to re-differentiate toward the chondrogenic lineage, while a substrate coated with a ceramic-based material (OS) reduced chondrocyte re-differentiation ability and enhanced the capacity of the cells to enter the osteogenic lineage. Moreover, we found that the marked changes in AHAC differentiation ability induced by expansion on OS were related to large differences in the expression of specific surface molecules, as compared to PL-expanded cells.

Coating of PL with COL and FN did not alter the expression of genes typically used to characterize the stage of chondrocyte differentiation (i.e., collagen types I and II), in agreement with the results presented by Brodkin et al. [2004] using bovine articular chondrocytes. However, COL-expanded AHAC displayed an increased expression of the hyaluronan receptor CD44, a surface molecule highly expressed during mesenchymal cell condensation in the early phases of chondrogenesis [Rousche and Knudson, 2002]. Since CD44 has also been postulated to be essential for proper cartilage matrix assembly [Knudson et al., 1999], its increased expression could be related to the higher chondrogenic capacity of AHAC expanded on COL.

AHAC expanded on OS expressed markedly lower collagen type II and higher BSP mRNA levels than PL-expanded cells, possibly indicating that cells acquired a preferential commitment toward the osteogenic lineage. In addition, OS-expanded cells displayed a reduced expression (i) of the protease CD26, whose decreased expression by chondrocytes has been associated with the progressive worsening of osteoarthritic lesions [Lapadula et al., 1995], and (ii) of the PDGF receptor CD140a, which by interacting with its ligands has been reported to be an important mediator of limb bud chondrogenesis [Ataliotis, 2000]. Lower expression levels of these membrane proteins could be related to the reduced chondrogenic capacity of AHAC expanded on OS. Following culture in osteogenic medium, all cells expressed levels of OC mRNA approaching those of osteoblasts. However, only OS-expanded cells upregulated expression of BSP, a marker previously reported to more specifically reflect osteoblastic differentiation [Satomura et al., 2000; Frank et al., 2002]. The highly variable mRNA expression of collagen type X, in conjunction with the negligible levels of collagen type II, indicate that the phenotype of cells following osteogenic induction was not typical of hypertrophic chondrocytes.

The most relevant finding of our study is that the substrates used for expansion "imprinted" AHAC and differentially primed their subsequent differentiation in response to lineage-specific stimuli. The same paradigm was previously reported using human bone marrow-derived mesenchymal progenitor cells (MPC). In particular, Mauney et al. recently demonstrated that plastic coating with denatured collagen supports expansion of MPC with increased osteogenic [Mauney et al., 2004] and adipogenic capacity [Mauney et al., 2005], thus proposing the use of specific substrates as a tool for progenitor cell expansion in cell-based tissue engineering approaches. Similarly, our results would prompt for the development of composite scaffolds for osteochondral tissue repair with a superficial membrane composed of a type II collagen material, overlaying a ceramic-based structure. In this context, it will be interesting to determine whether chondrocytes growing at the interface of such composites would acquire an intermediate phenotype, typical of cells at the tidemark of articular cartilage.

The mechanisms by which expansion on COL and OS modulate the post-expansion differentiation capacity of AHAC are not understood. AHAC could be activated by direct interactions with the specific substrate and/or by several "indirect" mediators (e.g., collagen type II fragments, Ca^{++} ions, or selective adsorption of specific serum proteins). In addition, it still has to be determined whether the superior chondrogenic or osteogenic differentiation ability of AHAC cultured respectively on COL or OS could be due to the selection of cell subpopulations with pre-existing commitment toward a specific lineage.

The process of chondrocyte de-differentiation by monolayer expansion and of subsequent re-differentiation in micromass cultures has been proposed as a model of cartilage repair following degeneration [Jakob et al., 2004]. In fact, monolayer-expanded chondrocytes display gene expression profiles resembling those of osteoarthritic (OA) cells [Sandell and Aigner, 2001; Stokes et al., 2002], and following 3D culture they can partially re-differentiate and deposit hyaline-like ECM [Jakob et al., 2001; Risbud and Sittinger, 2002; Grad et al., 2003; Barbero et al., 2004]. In this context, it would be tempting to speculate that pellet cultures of AHAC expanded on COL or OS mimic the processes of cartilage repair at different stages of OA. At early stages of OA, following derangement of the pericellular matrix, chondrocytes would be directly in contact with collagen type II fibrils, still predominantly intact; at this phase, in the attempt to repair the initial degeneration, cells are known to become activated, expressing increased levels of collagen type II and GAG [Aigner and Dudhia, 1997; Aigner and Stove, 2003]. These synthetic profiles resemble those observed by COL-expanded AHAC following culture in pellets. Conversely, at late stages of OA, following denudation of subchondral bone, chondrocytes would be in direct contact with calcium phosphate particles; at this phase, cells are reported to acquire a fibroblastic morphology and express elevated levels of collagen type I and BSP, resulting in a mostly fibro-cartilaginous repair tissue [de Bri et al., 1997]. These phenotypic and synthetic profiles resemble those observed by OS-expanded AHAC following culture in pellets. Further studies are clearly required to validate the relevance of the proposed experimental systems as stage-specific models of the OA disease.

In conclusion, we have demonstrated that expansion of AHAC on substrates coated with collagen type II or calcium phosphate improves the capacity of chondrocytes to differentiate respectively toward the chondrogenic or osteogenic lineage. The specific modulation of cell commitment induced by expansion on these substrates could help understanding pathophysiological mechanisms of cartilage degeneration/repair and support the selection of appropriate scaffold compositions for the repair of osteochondral defects.

ACKNOWLEDGMENTS

We are grateful to Millenium Biologix (Schlieren, CH) for the generous supply of BioCoat™ Osteologic™ slides. We thank Miss Chantal Pauli's kind assistance in the immunohistochemistry procedure and the support of Dr. Jose Diaz-Romero in the flow cytometry assessments. This work was supported financially by the Swiss National Science Foundation, Research Program 46 "Implants and Transplants" (grant no. 4046-58623).

REFERENCES

Aigner T, Dudhia J. 1997. Phenotypic modulation of chondrocytes as a potential therapeutic target in osteoarthritis: a hypothesis. Ann Rheum Dis 56:287–291.

Aigner T, Stove J. 2003. Collagens-major component of the physiological cartilage matrix, major target of cartilage degeneration, major tool in cartilage repair. Adv Drug Deliv Rev 55:1569–1593.

Ataliotis P. 2000. Platelet-derived growth factor A modulates limb chondrogenesis both in vivo and in vitro. Mech Dev 94:13–24.

Barbero A, Ploegert S, Heberer M, Martin I. 2003. Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes. Arthritis Rheum 48: 1315–1325.

Barbero A, Grogan S, Schafer D, Heberer M, Mainil-Varlet P, Martin I. 2004. Age related changes in human articular chondrocyte yield, proliferation and post-expansion chondrogenic capacity. Osteoarthritis Cartilage 12:476–484.

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.

Brodkin KR, Garcia AJ, Levenston ME. 2004. Chondrocyte phenotypes on different extracellular matrix monolayers. Biomaterials 25:5929–5938.

de Bri E, Lei W, Reinholt FP, Mengarelli-Widholm S, Heingard D, Svensson O. 1997. Ultrastructural immunolocalization of bone sialoprotein in guinea-pig osteoarthritis. Osteoarthritis Cartilage 5:387–393.

Dell'Accio F, Vanlauwe J, Bellemans J, Neys J, De Bari C, Luyten FP. 2003. Expanded phenotypically stable chondrocytes persist in the repair tissue and contribute to cartilage matrix formation and structural integration in a goat model of autologous chondrocyte implantation. J Orthop Res 2003; 21:123–131.

Diaz-Romero J, Gaillard JP, Grogan SP, Nesic D, Trub T, Mainil-Varlet P. 2005. Immunophenotypic analysis of human articular chondrocytes: Changes in surface markers associated with cell expansion in monolayer culture. J Cell Physiol 202:731–742.

Farndale RW, Buttle DJ, Barrett AJ. 1986. Improved quantitation and discrimination of sulphated glycosami-

noglycans by use of dimethylmethylene blue. Biochim Biophys Acta 883:173–177.

Frank O, Heim M, Jakob M, Barbero A, Schafer D, Bendik I, Dick W, Heberer M, Martin I. 2002. Real-time quantitative RT-PCR analysis of human bone marrow stromal cells during osteogenic differentiation in vitro. J Cell Biochem 85:737–746.

Grad S, Kupcsik L, Gorna K, Gogolewski S, Alini M. 2003. The use of biodegradable polyurethane scaffolds for cartilage tissue engineering: Potential and limitations. Biomaterials 24:5163–5171.

Grogan SP, Rieser F, Winkelmann V, Berardi S, Mainil-Varlet P. 2003. A static, closed and scaffold-free bioreactor system that permits chondrogenesis in vitro. Osteoarthritis Cartilage 11:403–411.

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.

Jakob M, Demarteau O, Schafer D, Hintermann B, Dick W, Heberer M, Martin I. 2001. Specific growth factors during the expansion and redifferentiation of adult human articular chondrocytes enhance chondrogenesis and cartilaginous tissue formation in vitro. J Cell Biochem 81:368–377.

Jakob M, Demarteau O, Suetterlin R, Heberer M, Martin I. 2004. Chondrogenesis of expanded adult human articular chondrocytes is enhanced by specific prostaglandins. Rheumatology (Oxford) 43:852–857.

Kino-Oka M, Yashiki S, Ota Y, Mushiaki Y, Sugawara K, Yamamoto T, Takezawa T, Taya M. 2005. Subculture of chondrocytes on a collagen type I-coated substrate with suppressed cellular dedifferentiation. Tissue Eng 11: 597–608.

Knudson CB, Nofal GA, Pamintuan L, Aguiar DJ. 1999. The chondrocyte pericellular matrix: A model for hyaluronan-mediated cell-matrix interactions. Biochem Soc Trans 27:142–147.

Langstaff S, Sayer M, Smith TJ, Pugh SM, Hesp SA, Thompson WT. 1999. Resorbable bioceramics based on stabilized calcium phosphates. Part I: Rational design, sample preparation and material characterization. Biomaterials 20:1727–1741.

Lapadula G, Iannone F, Zuccaro C, Covelli M, Patella V, Lobianco G, Pipitone V. 1995. Expression of membrane-bound peptidases (CD10 and CD26) on human articular chondrocytes. Possible role of neuropeptidases in the pathogenesis of osteoarthritis. Clin Exp Rheumatol 13: 143–148.

Mauney JR, Kaplan DL, Volloch V. 2004. Matrix-mediated retention of osteogenic differentiation potential by human adult bone marrow stromal cells during ex vivo expansion. Biomaterials 25:3233–3243.

Mauney JR, Volloch V, Kaplan DL. 2005. Matrix-mediated retention of adipogenic differentiation potential by human adult bone marrow-derived mesenchymal stem cells during ex vivo expansion. Biomaterials 26:6167–6175.

Risbud MV, Sittinger M. 2002. Tissue engineering: Advances in in vitro cartilage generation. Trends Biotechnol 20:351–356.

Rousche KT, Knudson CB. 2002. Temporal expression of CD44 during embryonic chick limb development and modulation of its expression with retinoic acid. Matrix Biol 21:53–62.

Sandell LJ, Aigner T. 2001. Articular cartilage and changes in arthritis. An introduction: Cell biology of osteoarthritis. Arthritis Res 3:107–113.

Satomura K, Krebsbach P, Bianco P, Gehron RP. 2000. Osteogenic imprinting upstream of marrow stromal cell differentiation. J Cell Biochem 78:391–403.

Scully SP, Lee JW, Ghert PA, Qi W. 2001. The role of the extracellular matrix in articular chondrocyte regulation. Clin Orthop Relat Res 391 (Suppl):S72–S89.

Shakibaei M, De Souza P, Merker HJ. 1997. Integrin expression and collagen type II implicated in maintenance of chondrocyte shape in monolayer culture: An immunomorphological study. Cell Biol Int 21:115–125.

Stokes DG, Liu G, Coimbra IB, Piera-Velazquez S, Crowl RM, Jimenez SA. 2002. Assessment of the gene expression profile of differentiated and dedifferentiated human fetal chondrocytes by microarray analysis. Arthritis Rheum 46:404–419.

Tallheden T, Dennis JE, Lennon DP, Sjogren-Jansson E, Caplan AI, Lindahl A. 2003. Phenotypic plasticity of human articular chondrocytes. J Bone Joint Surg Am 85(Suppl):93–100.

van der Kraan PM, Buma P, van Kuppevelt T, van den Berg WB. 2002. Interaction of chondrocytes, extracellular matrix and growth factors: Relevance for articular cartilage tissue engineering. Osteoarthritis Cartilage 10: 631–637.