Chondrogenic Differentiation of Bovine Bone Marrow Mesenchymal Stem Cells (MSCs) in Different Hydrogels: Influence of Collagen Type II Extracellular Matrix on MSC Chondrogenesis

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Abstract: Bone marrow mesenchymal stem cells (MSCs) are candidate cells for cartilage tissue engineering. This is due to their ability to undergo chondrogenic differentiation after extensive expansion in vitro and stimulation with various biomaterials in three-dimensional (3-D) systems. Collagen type II is one of the major components of the hyaline cartilage and plays a key role in maintaining chondrocyte function. This study aimed at analyzing the MSC chondrogenic response during culture in different types of extracellular matrix (ECM) with a focus on the influence of collagen type II on MSC chondrogenesis. Bovine MSCs were cultured in monolayer as well as in alginate and collagen type I and II hydrogels, in both serum free medium and medium supplemented with transforming growth factor (TGF) β1. Chondrogenic differentiation was detected after 3 days of culture in 3-D hydrogels, by examining the presence of glycosaminoglycan and newly synthesized collagen type II in the ECM. Differentiation was most prominent in cells cultured in collagen type II hydrogel, and it increased in a time-dependent manner. The expression levels of the of chondrocyte specific genes: sox9, collagen type II, aggrecan, and COMP were measured by quantitative ''Real Time'' RT-PCR, and genes distribution in the hydrogel beads were localized by in situ hybridization. All genes were upregulated by the presence of collagen, particularly type II, in the ECM. Additionally, the chondrogenic influence of TGF β1 on MSCs cultured in collagen-incorporated ECM was analyzed. TGF β1 and dexamethasone treatment in the presence of collagen type II provided more favorable conditions for expression of the chondrogenic phenotype. In this study, we demonstrated that collagen type II alone has the potential to induce and maintain MSC chondrogenesis, and prior interaction with TGF β1 to enhance the differentiation.

Keywords: mesenchymal stem cells (MSC); chondrogenic differentiation; extracellular matrix; collagen type II

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INTRODUCTION

Stem cells are undifferentiated cells that are defined by their ability, at the single cell level, to self-renew and to differentiate to produce mature progeny cells, which include both nonrenewing progenitors and terminally differentiated effectors (Wagers and Weissman, 2004). Bone marrow mesenchymal stem cells (MSCs) are currently among the best characterized adult stem cells (Majumdar et al., 1998; Pittenger et al., 1999) isolated from various tissue sources such as fat, muscle, and bone (Jiang et al., 2002b; Sottile et al., 2002; Zuk et al., 2002). These cells are able to differentiate into bone, fat, cartilage, muscle tissue, and neurons (Mackay et al., 1998; Reyes et al., 2001; Sekiya et al., 2004; Woodbury et al., 2000) and were ultimately reported in almost all cell lineages (Jiang et al., 2002; Krause et al., 2001). For these reasons, the isolation and manipulation of adult stem cells represents a promising tool for understanding tissue development and regeneration, as well as for studying the engineered repair of tissues and organs (Orlic et al., 2001; Pittenger and Martin, 2004; Wakitani et al., 2002).

The extracellular molecular signaling pathways are being actively investigated. Extracellular signaling involves the interaction of cell surface receptors with soluble cytokines and growth factors, with the extracellular matrix (ECM),

such as collagen and proteoglycans, or with the surface proteins of the neighboring cells (Heng et al., 2004).

In addition to maintaining the structural integrity of the tissues, the ECM has physiological functions, such as passing nutrients to the cells, acting as a reservoir for physiological mediators, and mediating cellular functions through interaction with cell surface receptors (Comper, 1996). The integrins are a major family of ECM receptors that transmit information from the matrix to the cells, thereby playing a key role in the regulation of cell survival, proliferation, differentiation, and matrix remodeling (Loeser, 2002).

Articular cartilage is an avascular tissue composed of chondrocytes and ECM, which predominantly consists of collagen type II, aggrecan, and hyaluronic acid. In addition to these macromolecules, collagen types VI, IX, XI; decorin; biglycan, cartilage oligomeric matrix protein (COMP); and fibromodulin are also present in minor concentrations (Muir, 1995; Poole et al., 2001). It is well known that damaged articular cartilage has poor intrinsic regenerative capacity. Cell therapy using chondrocytes or their precursors embedded in some biocompatible scaffold is a possible approach for hyaline cartilage reconstruction. Such three-dimensional (3-D) support is required for articular chondrocytes to maintain their differentiated phenotype in vitro or for the MSC to undergo chondrogenesis (Lemare et al., 1998). Materials such as agarose, collagen, fibrin, alginate, and biopolymers have all been used as 3-D scaffolds for chondrocytes and MSC cultures (Batorsky et al., 2005; Huang et al., 2004; Kavalkovich et al., 2002; Mueller and Glowacki, 2001; Ponticiello et al., 2000; Wayne et al., 2005).

In order to induce MSC chondrogenesis, factors that support strong cell–cell interaction, growth factors, and an environment that maintains spherical morphology, such as polymer gels, are all important. Strong cell–cell interaction mediated by cell adhesion molecules such as *N*-cadherin and integrins allow MSC conversion to prechondroblasts at the precartilage mesenchymal condensation stage during limb development (Haas and Tuan, 1999; Hall and Miyake, 1995). Various bioactive factors, such as fibroblast growth factor, transforming growth factors (TGF) β1 and β3, bone morphogenic proteins (BMPs)-2, -6, and -9, and insulin-like growth factors, have been reported to induce or maintain chondrogenesis (Indrawattana et al., 2004; Muraglia et al., 2000; Sekiya et al., 2002).

Although there has been an increase in information regarding the role of growth factors and cytokines as inducers and mediators of MSC differentiation, little is known about the influence of ECM on MSC chondrogenic differentiation. Even less is known regarding the nature of communication and the mechanism of interaction between the MSCs and the cartilaginous matrix during embryogenesis or homeostasis of articular cartilage.

The goals of this study were to analyze the influence of different types of ECM, particularly collagen type II, on MSC chondrogenesis with or without interaction with the chondrogenic growth factor TGF β1. Ultimately, these studies may contribute to a better understanding of the development, physiology, and regeneration of articular cartilage.

MATERIALS AND METHODS

Harvest and Isolation of Bovine MSC

Bone marrow was aspirated from four calves (2 months old), and MSCs were isolated by previously described methods (Bosnakovski et al., 2004, 2005). Briefly, the bone marrow sample was washed twice with phosphate buffered saline (PBS), and twice again with Dulbecco's Modified Eagle Medium (DMEM; GIBCO BRL, Grand Islands, NY). After determination of the cell viability and number, $5 \times 10^4/cm^2$ nucleated cells were plated in medium that consisted of DMEM (low glucose) containing penicillin G 100 U/mL, streptomycin 100 μg/mL, amphotericin B 0.25 μg/mL, HEPES 2.4 mg/mL, $NaHCO_3$ 3.7 mg/mL, and 10% fetal bovine serum (FBS; lot No. 5300C, ICN, Biomedicals, Aurora, OH), and cultured at 37°C in a humidified atmosphere containing 5% CO_2 until the second passage. Experiments were performed with at least six different isolated cell lines.

Hydrogel Preparation

Alginate and collagen type I and II hydrogels were prepared using a combination of previously reported protocols (Elisseeff et al., 2002; Qi and Scully, 2002). An alginate solution (1.5%) prepared from low-viscosity sodium alginate (Wako Pure Chemical Co., Osaka, Japan) in 0.15 M NaCl was sterilized by filtering through a 0.45 μm filter. For preparation of collagen type I or II hydrogels, 700 μL of collagen type I or II solutions (0.3%, Koken Co., Tokyo, Japan) were neutralized with 100 μL HEPES buffer and mixed with 280 μL 1.5% alginate solution. A minimum of 35% alginate in the mixture was necessary to obtain a solid hydrogel. Cells from the second or third passage were harvested with 0.25% trypsin in 0.02% EDTA, and then divided in three parts. Each part was mixed with one of the hydrogels to a final concentration of $1–2 \times 10^6$ cells/mL solution. Cells/hydrogel suspension was slowly dropped into 102 mM $CaCl_2$ using a pipette tip (2–200 μL). After initial gelatinization, the beads were allowed to polymerize for 10 min. Subsequently, they were washed three times with 0.15 M NaCl and once with DMEM and were cultured overnight at 37°C/5% CO_2. The polymerized alginate solution formed compact spherical beads of 2–3 mm diameter. Collagen type I and II hydrogels had a more discoid shape, approximately 2×4 mm in size. They could not polymerize to typical beads prior to contact with $CaCl_2$ because of the low alginate concentrations. On the following day, the beads were transferred to six-well cell culture plates (16 beads/well), and they were cultured in plain or chondrogenic medium for 21 days. The plain medium was a serum-free chemically defined medium consisting of DMEM (high glucose), insulin 6.25 μg/mL, transferin 6.25 μg/mL, selenious acid 6.25 μg/mL, bovine serum

albumin 1.25 mg/mL, pyruvate 1 mM, linoleic acid 5.35 μg/mL, and ascorbate 2-phosphate 50 μg/mL (all from Sigma Chemical Co., St. Louis, MO). The chondrogenic medium was plain medium supplemented with 10 ng/mL TGF β1 (R & D Systems, Minneapolis, MN) and 100 nM dexamethasone (Sigma Chemical Co.).

MSCs cultured in monolayers were used as a control, for comparing the influence of the type of culture system (monolayer and 3-D) on chondrogenesis. Aliquots of the cells used for hydrogel preparation were plated (5×10^3 cells/cm^2) in six-well plates and cultured in plain and chondrogenic medium under conditions similar to those of cells in hydrogels.

Histological and Immunohistochemical Analysis of the Hydrogel Beads

Samples were harvested at the beginning of the experiment (day 0) and after 3, 6, and 21 days of culture. They were fixed in 10% neutral buffered formalin containing 0.1 M $CaCl_2$ for 2 days and embedded in paraffin. Hematoxylin and eosin (H & E) staining of paraffin sections (4 μm) was performed to evaluate cell morphology in the beads. Sulfated glycosaminoglycans (GAGs) were visualized by staining with 0.5% alcian blue (pH 1.0) for 10 min. Newly synthesized collagen type II was detected using polyclonal rabbit anti-bovine antibody (LSL Co., Tokyo, Japan). Briefly, after deparaffinization, the sections were predigested with 2.5% hyaluronidase (Type I–S, Sigma Chemicals Co.) for 30 min at 37°C to facilitate antibody access. Endogenous peroxidase was quenched by treatment with 0.3% H_2O_2 in methanol at room temperature for 30 min, and nonspecific antibody binding was blocked by incubating sections in 10% normal goat serum at 37°C for 30 min. Primary antibody was diluted 1:500 in 0.05 M Tris buffer (pH 7.6) and applied overnight at 4°C. Sections were then incubated with the secondary antibody, swine anti-rabbit immunoglobulin (DAKO, Glostrup, Denmark), for 60 min, followed by detection with the rabbit PAP kit (DAKO). Collagen type II was visualized by reactions with 0.05% diaminobenzidine containing 0.01% H_2O_2.

In Situ Hybridization

For in situ hybridization, hydrogel beads harvested on day 0, 3, 6, and 21 were embedded in OCT embedding medium (Tissue-Tek; Sakura Finetechnical Co., Tokyo, Japan) and frozen in liquid nitrogen. Frozen sections (10 μm) were cut and mounted on silane-coated glass slides. Two kinds of nonoverlapping 45-m antisense oligonucleotide probes were synthesized to detect every mRNA of interest. The following genes sequences were used: for collagen type I, nucleotide residues 2408–2453 and 4108–4153 (Gen Bank AB008683); for collagen type II, nucleotide residues 278–323 and 777–822 (Gen Bank X02420); and for aggrecan, nucleotide residues 5540–5585 and 6750-6795 (Gen Bank U76615). Oligonucleotides were labeled with ^{35}S-adenosine thiotriphosphate to a specific activity of 0.5×10^9 dpm/μg DNA, using terminal deoxynucleotidyl transferase. Sections were fixed with 4% paraformaldehyde in 0.05 M Tris buffer (pH 7.6) for 15 min, and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min. Hybridization was performed at 42°C overnight by reaction with labeled oligonucleotide probes at a final radioactivity of 0.5×10^7 cpm/mL in 50% formamide, which contained 30 mM Tris-HCl (pH 7.4), 0.6 M NaCl, 1 mM EDTA, 100 mM dithiothreitol, 1× Denhardt's solution, 0.25% sodium dodecyl sulfate, 10% dextran sulfate, and 200 μg/mL yeast tRNA. Control hybridization was performed in the presence of 20-fold excess amount of unlabeled antisense probe. Slides were washed in 2× SSC containing 0.1% sarkosyl for 30 min at room temperature, followed by washing twice in 0.1× SSC/0.1% sarkosyl for 40 min at 55°C. Slides were dipped into autoradiographic emulsion (NTB-2, Kodak, Rochester, NY), exposed at 4°C for 3 weeks, and counterstained with hematoxylin.

Isolation of RNA From the Hydrogels and cDNA Synthesis

On days 0, 3, 6, and 21, beads ($n = 6–8$) were transferred to 1.5 mL centrifuge tubes and dissolved with 1 mL reconstruction buffer (50 mM EDTA and 10 mM HEPES in 0.15 M NaCl) for 10 min at 37°C. The cell suspension was centrifuged (200*g*) and the cell pellets were washed once with PBS. Total RNA from reconstructed cells and cells from the monolayer was isolated using Trizol (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Samples were treated with DNase to remove possible genomic DNA contamination. Subsequently, first-strand cDNA was synthesized in a 20 μL reaction mixture from 2 μg total RNA using M-MLV reverse transcriptase (Invitrogen) with oligo $(dT)_{20}$ as a primer. The sample that did not contain transcription enzyme in the reaction mixture was used as the negative control for checking genomic contamination.

To compare level of MSC chondrogenesis and for positive control in PCR reaction, total RNA was harvested from bovine chondrocytes that were isolated by collagenase digestion of articular cartilage pieces from the same donors as those for bone marrow (Bosnakovski et al., 2004).

Measurement of Gene Expression by a Quantitative "Real Time" RT-PCR (qRT-PCR) Method

The amount of cDNA was measured by a qRT-PCR method using the Smart Cycler System (Cepheid, CA). The PCR reaction was carried out in 25 μL final volume, which contained PCR buffer, 3 mM $MgCl_2$, 0.3 mM dNTP mixture, 0.3 μM each of bovine specific primer (Table I), and Taq polymerase (1.25 U/tube; Takara Biomedicals, Otsu, Japan). Thermal cycling was carried out for 45 cycles at 95°C for 5 s, 55–60°C for 15 s, and 75°C for 15 s. The amount of PCR product was estimated using absolute quantification method;

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Table I. Bovine marker genes used in quantitative "Real Time" RT-PCR (qRT-PCR).

Gene	Primer nucleotide sequence	Product size (bp)	Accession number
GAPDH	Forward 5′ CCT TCA TTG ACC TTC ACT ACA TGG TCT A Reverse 5′ TGG AAG ATG GTG ATG GCC TTT CCA TTG	127	U85042
Collagen type I	Forward 5′ TGC TGG CCA ACC ATG CCT CT Reverse 5′ CGA CAT CAT TGG ATC CTT GCA G	120	AB008683
Collagen type II	Forward 5′ ATC CAT TGC AAA CCC AAA GG Reverse 5′ CCA GTT CAG GTC TCT TAG AG	147	X02420
Collagen type X	Forward 5′ CAT GCT GCC ACA AAC AGC Reverse 5′ TGG ATG GTG GGC CTT TTA	110	X53556
Aggrecan	Forward 5′ CAC TGT TAC CGC CAC TTC CC Reverse 5′ GAC ATC GTT CCA CTC GCC CT	303	U76615
Sox9	Forward 5′ CAT GAA GAT GAC CGA CGA G Reverse 5′ CGT CTT CTC CGT GTC GGA	118	AF278703
COMP	Forward 5′ TTC GGA ACG CAC TGT GG Reverse 5′ TGC AGG AAC CAG CGG TA	112	X74326

by measuring the intensity of the fluorescence of SYBR Green I interacted into the PCR product and compared to the standard curve of every gene, separately. The mRNA expression levels of target genes were normalized by dividing their value by the value of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level. The quality of the PCR products was checked by melting curve analysis, electrophoresis, and sequencing.

Statistical Analyses

Data were evaluated from at least six independent experiments and are represented as mean ± SD. One-Way ANOVA followed by multiple pairwise comparisons was performed to determine differences among the groups. The results were considered significantly different at $P < 0.05$.

RESULTS

Histological and Immunohistochemical Determination of the Chondrogenesis

To evaluate the effect of various ECM components on chondrogenesis, we cultured bovine MSCs in hydrogels composed of alginate, collagen type I and II. Structural organization and cell proliferation, deposition of sulfated GAG, and the presence of newly synthesized collagen type II in hydrogels were analyzed on days 0, 3, 6, and 21, by H & E, alcian blue, and immunohistochemical staining, respectively, (Fig. 1). Homogeneous cell distribution throughout the bead was observed in all types of hydrogels. During the culture period, the cells proliferated in all types of cultures, with a notable increased proliferation rate in collagen hydrogels and in chondrogenic medium (data not shown). Cells maintained a round plump shape, characteristic of chondrocytes, and formed cell clusters during the culture period. Chondrogenesis was detected in all hydrogels, regardless of the type of culture medium (plain or chondrogenic). Detected GAG and collagen type II gradually increased during the culture period, and the difference in the staining intensity at day 0 and day 21 was obvious under all experimental conditions (Fig. 1). Among the various types of hydrogel and media, the chondrogenic markers were best expressed in cells cultured in collagen type II ECM and in chondrogenic medium, respectively. However, prior interpretation of the results it has to be considered that all three types of hydrogels have different physical properties (density, porosity, water contents, cells seeding efficiency), which can influence proliferation and differentiation ability of the cells.

In the present trial, MSCs from the second or third passage, independently, were used for the preparation of hydrogels. Cells from these two passages did not show any significant difference in the potential to undergo chondrogenic differentiation.

Quantitative Analyses of Chondrocyte-Related Gene Expression

Gene expression was analyzed by qRT-PCR in the differentiated cells produced by the various culture conditions (monolayer and 3-D culture), ECM (alginate and collagen types I and II), and culturing medium (plain serum-free and chondrogenic-TGF β1/dexamethasone supplemented medium). We assayed panel of chondrocyte-related genes: collagen type II (*coll 2*), aggrecan (*agg*), *COMP*, early chondrogenic transcription factor *sox9*, and a marker for hypertrophic chondrocytes, collagen type X (*coll 10*).

Cells cultured in monolayers in serum-free medium expressed only basal levels of *agg*, *coll 2*, and *coll 10* mRNA, but not of *sox9* and *COMP*. Conversely, cells cultured in 3-D systems, in plain medium, began to express or significantly increased the expression of chondrogenic genes just after 3 days (Figs. 2A and 3A). Sox9 showed markedly different expression between cells cultured in monolayers and in 3-D culture, being detection in all hydrogels, regardless of the medium (Fig. 4A). Sox9 is a member of the *Sox* (Sry-type HMG box) gene family, which are predominantly expressed in mesenchymal condensation and cartilage, and has been shown to activate type II collagen and aggrecan (Tsuchiya et al., 2003). We observed *coll 2*,

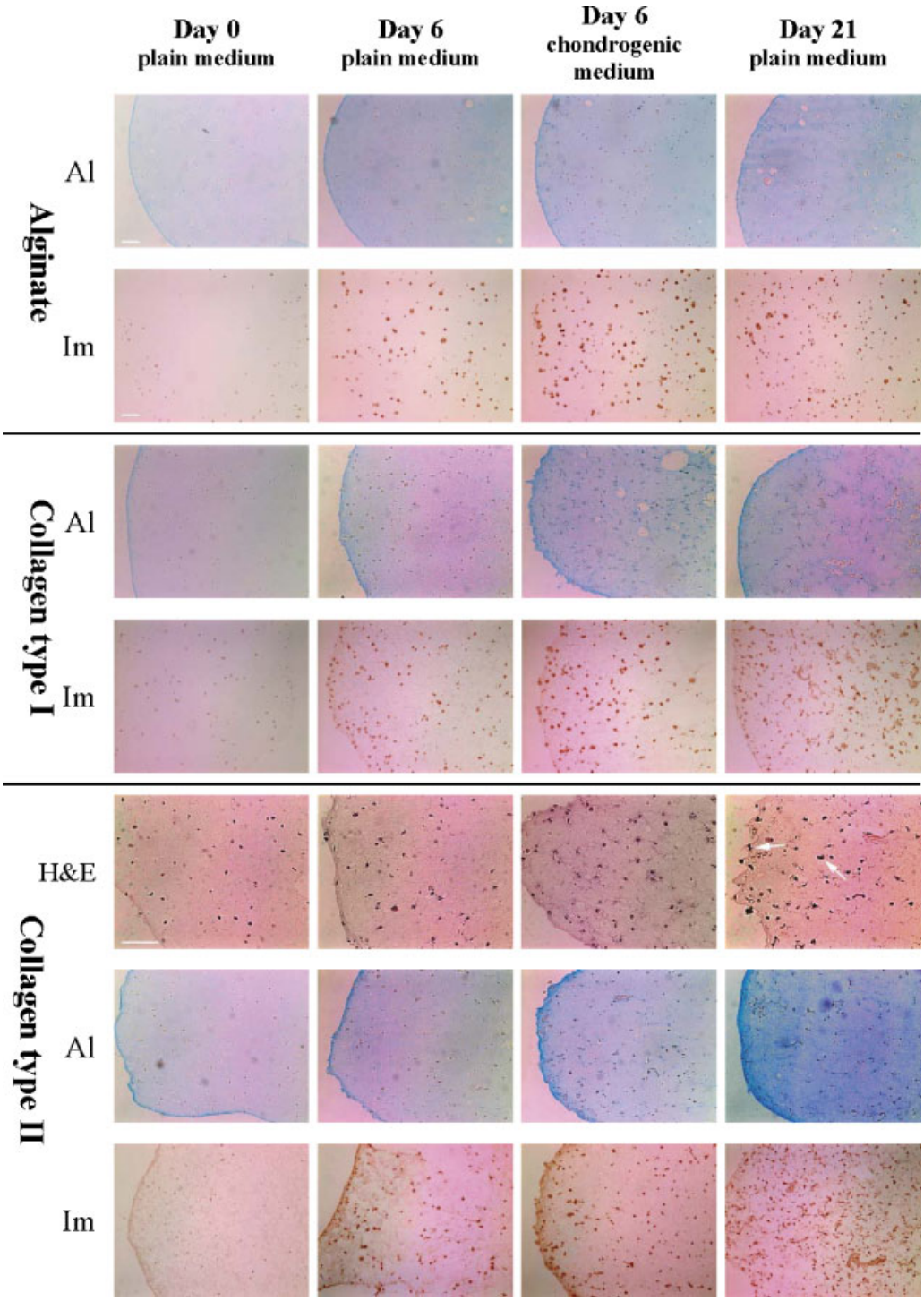

Figure 1. Mesenchymal stem cells (MSCs) cultured in alginate, and collagen types I and II hydrogels on days 0, 6, and 21 in serum-free and chondrogenic media, analyzed by hematoxylin and eosin (H & E), alcian blue (Al), and immunohistochemical staining (Im) for detection of type II collagen. Cells had a round, plump shape throughout the beads. Cell proliferation was obvious during the culture period, and on day 21, cells organized in clusters were noticeable (white arrows). Further, a significantly increased proliferation rate was detected in beads cultured in TGF β1 supplemented medium. Positive metachromatic staining and immunoreactivity for type II collagen was detected on day 3, and the intensity increased in a time-dependent manner. Scale bar 125 μm. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

agg, and *COMP* expression at a later time point in 3D culture, consistent with an early activating role of sox9. The levels of *coll 2* and *agg* mRNA continued to increase in collagen type I and II hydrogels from day 3 to day 21; however, they were maintained constant in alginate hydrogel.

In collagen type II hydrogel, from day 0 to day 6 and day 0 to day 21, *coll 2* increased 99- and 155-fold, and *agg* increased 12- and 31-fold, respectively (Figs. 2A and 3A). In alginate, on day 3, *coll 2* and *agg* were upregulated approximately 40-fold and threefold, respectively, and this

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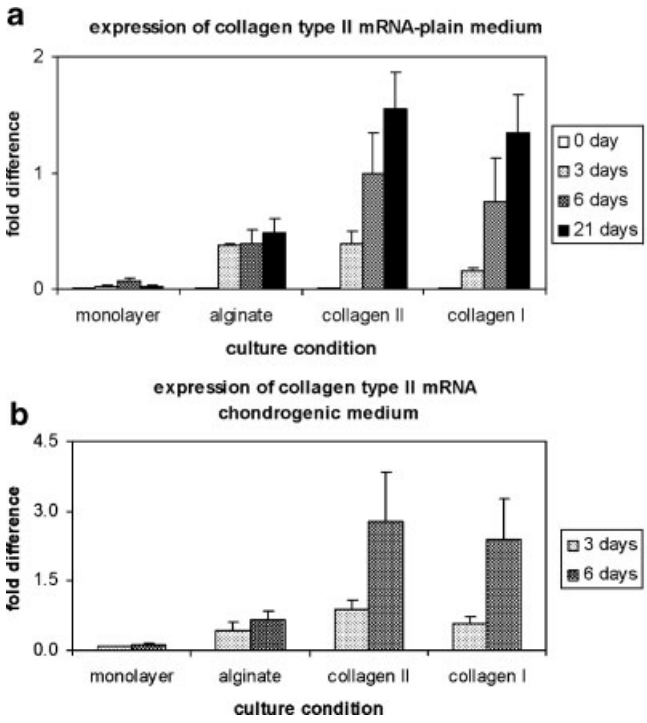

Figure 2. Quantitative analysis of collagen type II (*coll 2*) gene expression of MSCs cultured in plain (serum-free: (**a**)) and chondrogenic (TGF β1/ dexamethasone: (**b**) media. The expression level was normalized with respect to the expression level of GAPDH mRNA. The expression level of *coll 2* mRNA in the collagen type II hydrogel in plain medium on day 6 was represented as one. Data were evaluated from seven independent experiments and were represented as mean ± SD.

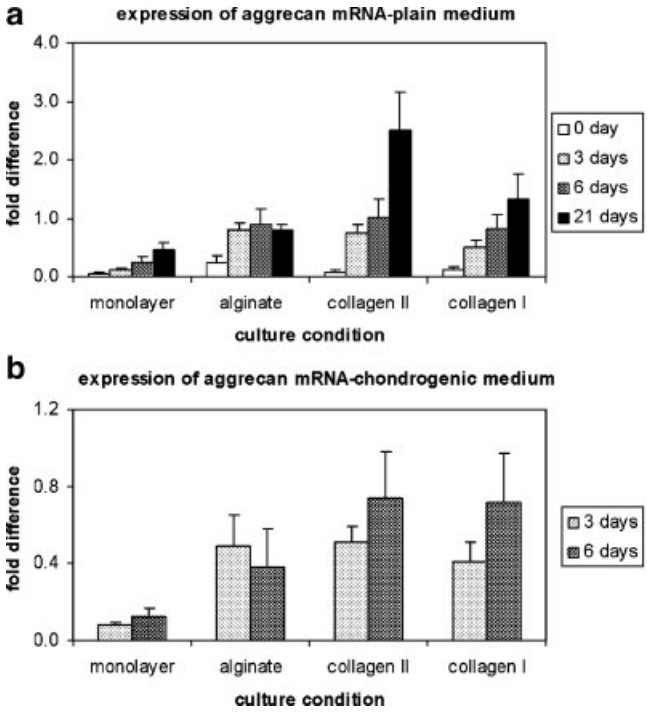

Figure 3. Quantitative analysis of aggrecan (*agg*) gene expression of MSCs cultured in plain (**a**) and chondrogenic (**b**) media. The expression level was normalized with respect to the expression level of GAPDH mRNA. The expression level of *agg* mRNA in the collagen type II hydrogel in plain medium on day 6 was represented as one. Data were represented as mean ± SD.

expression level remained constant during the culture period. Collagen types I or II incorporated in ECM significantly induced the expression of *coll 2* mRNA, and on day 21, it showed approximately 3.2-fold increase over that of alginate beads ($P < 0.05$). The expression of *agg* mRNA was also highest in the cells cultured in collagen type II, and on day 21 it showed 3.2- and 1.9-fold increase over that of alginate and collagen type I hydrogels ($P < 0.01$), respectively. In addition to the chondrogenic effect of the different 3-D cultures, we analyzed the influence of interaction among cells, ECM, and the potent bioactive factors, TGF β1 and dexamethasone on MSC differentiation. MSCs in monolayers stimulated by TGF β1 demonstrated increased but limited chondrogenic gene expression. The effect of this chondrogenic biofactor on gene expression was greatly enhanced in the 3-D culture system, followed by deposition of newly synthesized collagen type II in the ECM (Fig. 1). In the collagen type II hydrogel, on day 6, the *coll 2* mRNA was upregulated 2.8-fold in the chondrogenic medium in comparison with the plain medium ($P < 0.05$; Fig. 2). Interestingly, TGF β1 had a suppressive effect on *agg* mRNA, and on day 6, the expression decreased 30% in comparison with the plain medium (Fig. 3). A significant difference ($P < 0.01$) between the plain and chondrogenic media was found in all hydrogels, with approximately fivefold higher expression of *COMP* mRNA (Fig. 4B). COMP is one of the major noncollagenous proteins in cartilage and is reported to be an even more sensitive marker than collagen type II for the differentiation state of bovine articular chondrocytes (Zaucke et al., 2001). The expression of *sox9* and *coll 10* was also stimulated by TGF β1 (Fig. 4A and B); for example, in collagen type II ECM, after 6 days of culture, *coll 10* increased 1.8-fold in chondrogenic medium over plain medium ($P < 0.05$). Collagen type X is a marker for hypertrophic cartilage, which undergoes endochondral ossification and for articular chondrocytes that have become hypertrophic in osteoarthritis (Eerola et al., 1997; Gibson et al., 1997).

Additionally, we analyzed the influence of the ECM and the type of medium on the expression of *coll 1* mRNA in MSCs (Fig. 5). This gene is one of the markers for the de-differentiation of chondrocytes (Schulze-Tanzil et al., 2002). During culturing in monolayer, chondrocytes shift expression from *coll 2* to *coll 1*. MSCs cultured in monolayers and alginate cultures tended to increase *coll 1* expression (Fig. 5A). On the other hand, cells cultured for 21 days in collagen type I and particularly type II hydrogels showed a 2.3- and 2.8-fold decrease in expression compared with that of alginate ($P < 0.05$ and $P < 0.01$). In all culture conditions except collagen type I ECM, the *coll 1* mRNA was significantly ($P < 0.01$) downregulated by the chondrogenic medium, compared with the same experimental group cultured in plain medium.

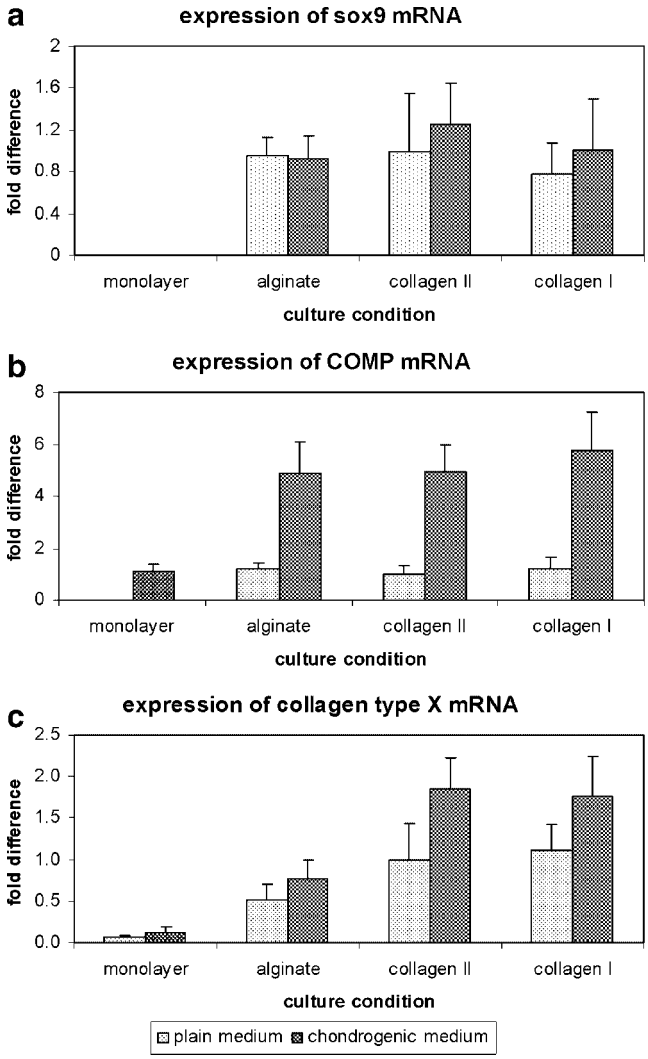

Figure 4. Quantitative analysis of sox9 (**a**), COMP (**b**), and collagen type X (*coll 10*; (**c**)) gene expression in MSCs cultured for 6 days. The expression level was normalized based on the GAPDH mRNA level. Expression levels of *sox9*, *COMP*, and *coll 10* mRNA in the collagen type II hydrogel in plain medium were each represented as one. Values were expressed as mean ± SD.

We compared gene expression level in chondrocytes produced in vitro (MSCs cultured for 6 days in collagen type II hydrogels and in chondrogenic medium) to that of primary isolated chondrocytes, cultured overnight in monolayers (Table II). While *coll 2*, *agg*, and *COMP* could be detected at responsibly similar levels, primary chondrocytes expressed very low to undetectable level of *sox9*, *coll 10*, and *coll 1*. Expression of *sox9* in freshly isolated chondrocytes could not be detected under equivalent RT-PCR conditions. However, with increasing sensitivity of the reactions, a low expression level of this gene was also detected (data not shown).

Localization of Gene Expression in the Hydrogels

In order to confirm the results from qRT-PCR and to localize mRNA expression in the hydrogel beads, we performed in situ hybridization for *coll 1*, *coll 2*, and *agg* mRNA (Figs. 6–8). The highest level of *coll 2* and *agg* expression

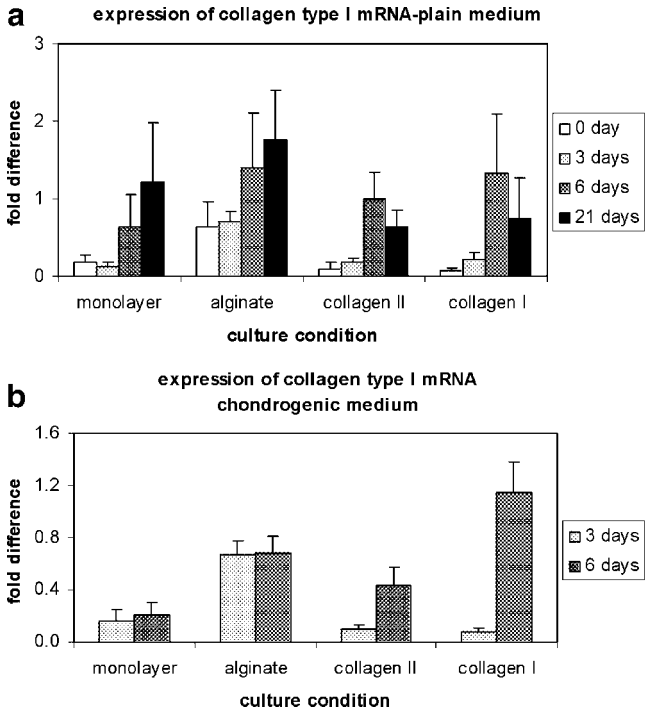

Figure 5. Quantitative analyses of collagen type I (*coll 1*) gene expression of MSCs cultured in plain (**a**) and chondrogenic (**b**) media. The expression level was normalized with respect to the expression level of GAPDH mRNA. The expression level of *coll 1* mRNA in the collagen type II hydrogel in plain medium on day 6 was represented as one. Data were represented as mean ± SD.

was detected in cells cultured in collagen type II ECM. The intensity of the signal increased with culture time and with the presence of TGF β1 in the medium. After 21 days of culture in plain medium, distribution of *coll 2* mRNA was evident in the center of the collagen bead. On the other hand, after short (6 days) treatment with TGF β1, *coll 2* was prominently detected in the cells at the periphery of the beads. This pattern of *coll 2* mRNA distributions was not notable in the alginate beads. Similar to *coll 2*, strong *coll 1* mRNA expression was detected in the center of the collagen type I hydrogel after 21 days of culture in plain medium. The incorporated collagen was most concentrated in the center of the beads where expression of chondrogenic-related genes

Table II. qRT-PCR analyses of chondrocyte-related gene expression in mesenchymal stem cells (MSCs) cultured for 6 days in collagen type II hydrogel and chondrogenic medium, and primary chondrocytes cultured overnight in 10% FBS-supplemented medium.

Gene	MSC	Chondrocytes
Collagen type II	1.00 ± 0.38	1.89 ± 0.21
Aggrecan	1.00 ± 0.33	1.70 ± 0.36
Sox9	1.00 ± 0.31	Not detected
COMP	1.00 ± 0.22	0.42 ± 0.07
Collagen type X	1.00 ± 0.21	0.03 ± 0.01
Collagen type I	1.00 ± 0.32	0.01 ± 0.003

The gene expression level in MSC was represented as 1 ± SD and that in chondrocytes was represented as fold difference ± SD.

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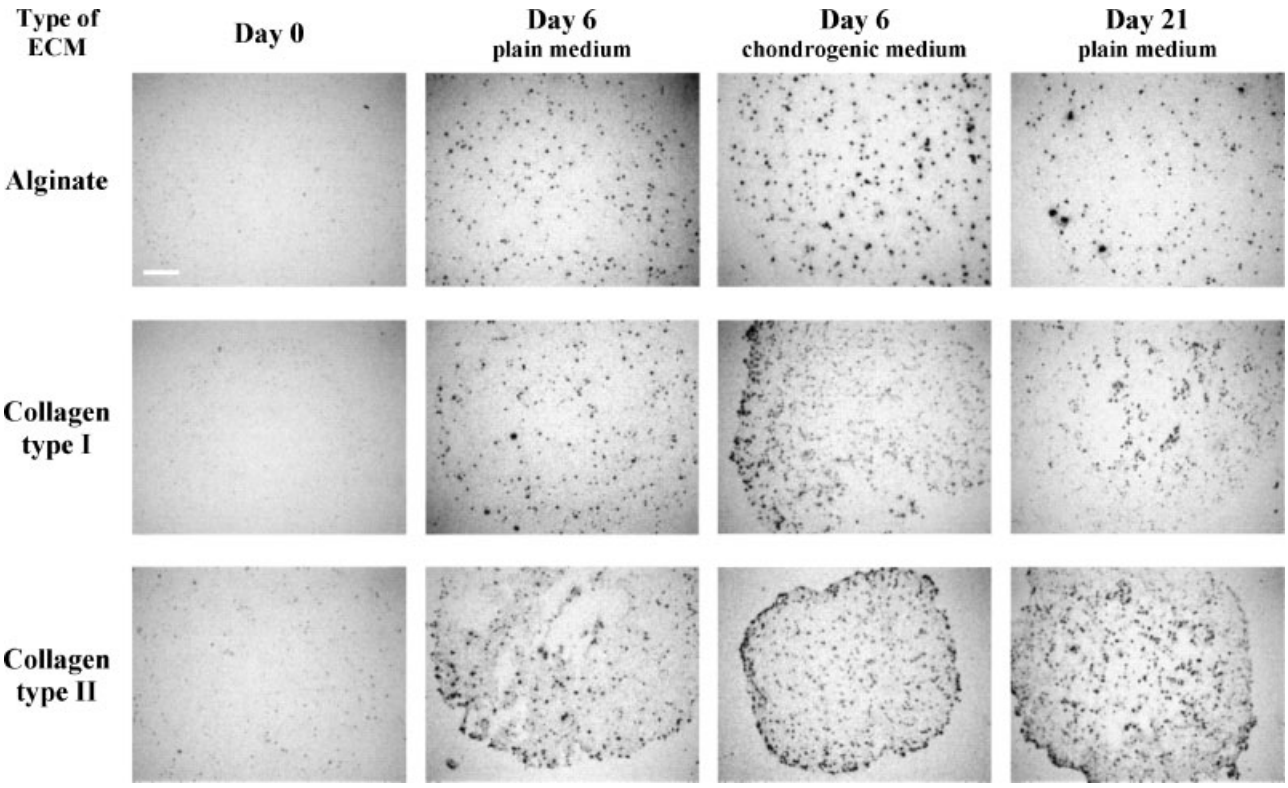

Figure 6. In situ hybridization of the alginate, collagen type I and II hydrogels on day 0, 6, and 21 for detection of *coll 2* mRNA. Scale bar 250 μm.

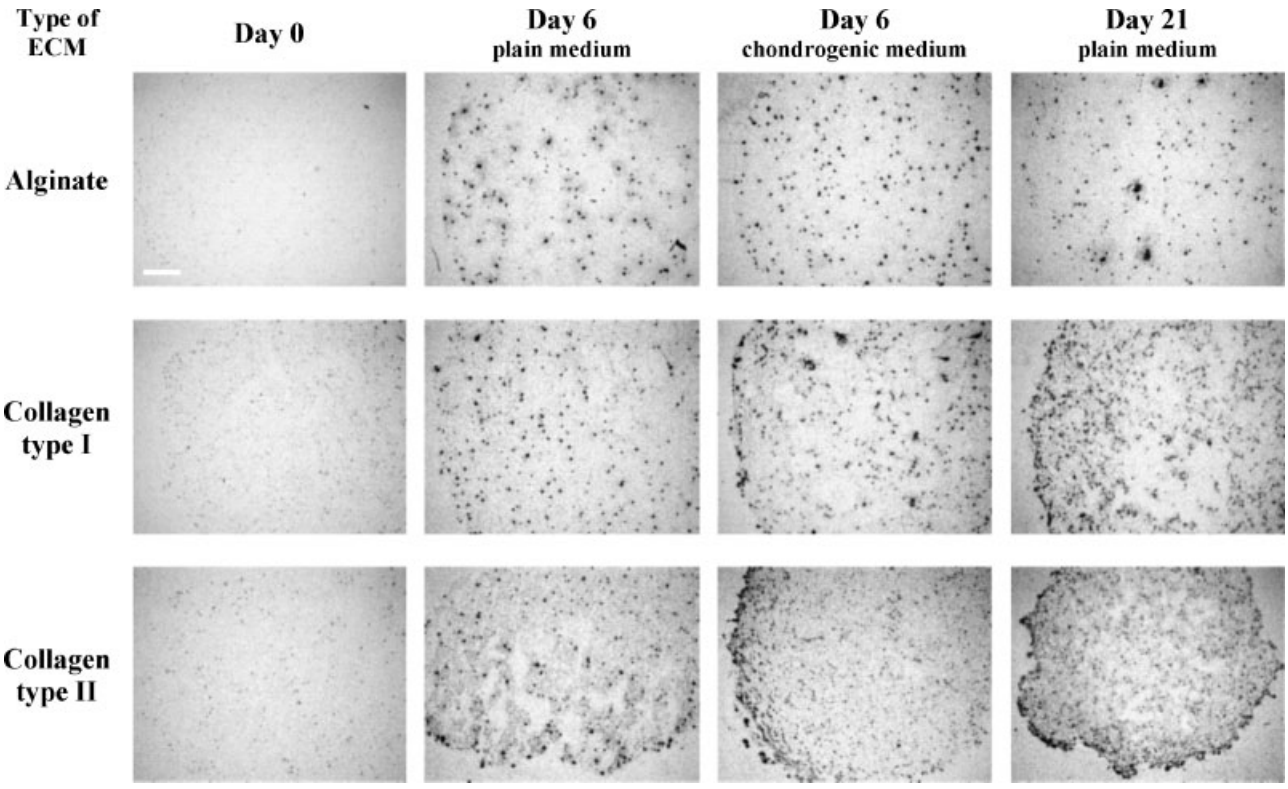

Figure 7. In situ hybridization of the alginate, collagen type I and II hydrogels on day 0, 6, and 21 for detection of *agg* mRNA. Scale bar 250 μm.

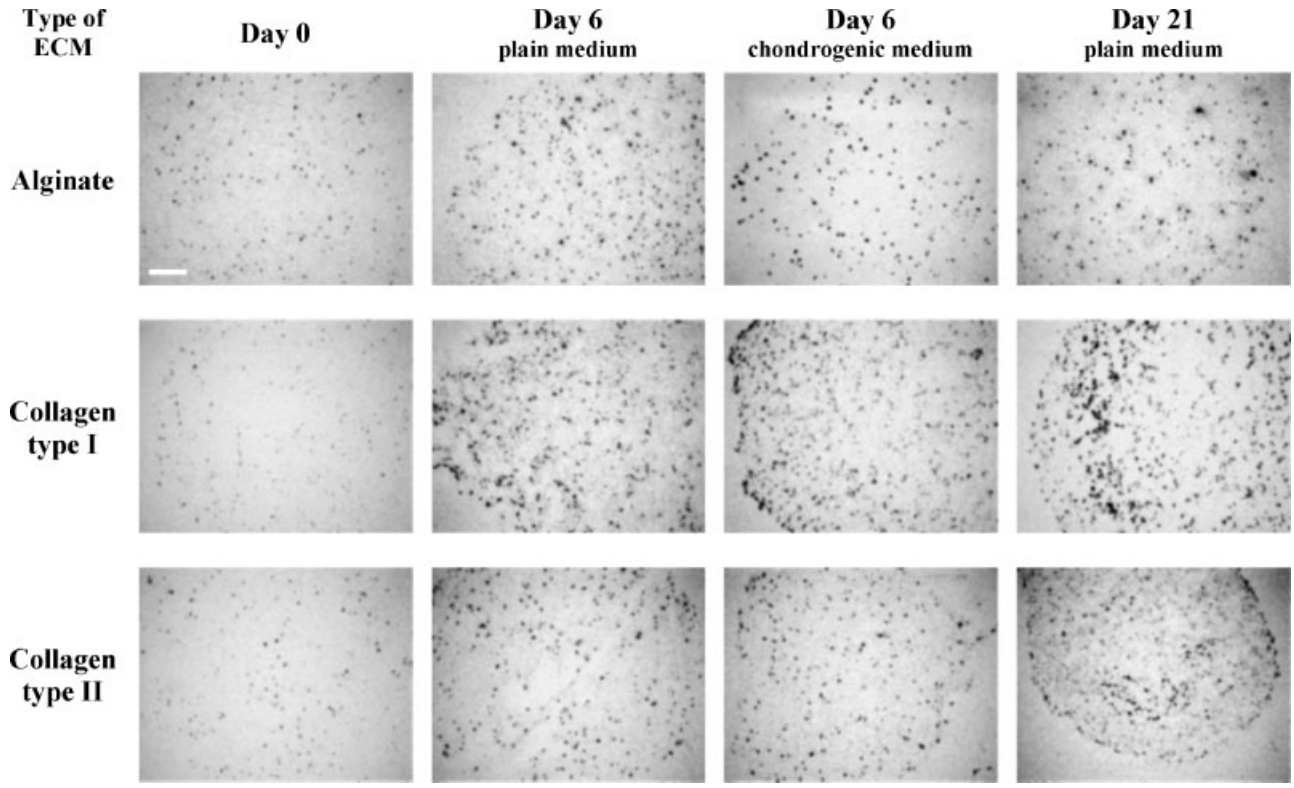

Figure 8. In situ hybridization of the alginate, collagen type I and II hydrogels on day 0, 6, and 21 for detection of *coll 1* mRNA. Scale bar 250 μm.

was the highest, confirming the previous suggestion that collagen was the dominant factor for MSC chondrogenesis.

DISCUSSION

By analyzing gene and protein expression, we have demonstrated the influence of various 3-D hydrogel cultures in inducing and maintaining MSC chondrogenic differentiation. Chondrogenesis of bovine MSCs occurred under serum-free condition and was greatly enhanced in media containing TGF β1. Among the different types of hydrogels, collagens type I and II showed the greatest degree of induced differentiation. Between the two types of collagen, type II was slightly better based on cell morphology and chondrogenic gene expression panel.

In order to resolve and understand the complexities of chondrogenesis, it is necessary to follow the process through different culture systems since each of these has certain limitations (Sekiya et al., 2002). Using a bovine experimental model, we have previously analyzed MSC chondrogenesis in monolayer cultures in response to TGF β1 stimulation (Bosnakovski et al., 2005). We also demonstrated that bovine MSC has the potential for self-inducing chondrogenic differentiation independent of any externally added biostimulants, if strong cell–cell interaction in the condensate pellet culture system is allowed (Bosnakovski et al., 2004). Having already determined the importance of strong cell–cell interaction and the influence of TGF β1 in MSC chondrogenesis, we proceeded to assess the influence of ECM on MSC chondrogenic differentiation.

It is well known that cells must interact with their microenvironment, from where soluble and insoluble signals trigger physiological responses through contact with the cell surface to maintain homeostasis. Various materials incorporated in 3-D culture systems have been shown to be useful tools in studying chondrogenesis, chondrocytes, and matrix biology (Qi and Scully, 2002; Wayne et al., 2005). In order to clarify the influence of the collagen ECM on MSC differentiation, we used two control groups. One was a monolayer culture and the other was a well-documented alginate hydrogel that allows certain macromolecules, such as collagen fibers, to be incorporated within it. Alginate is a polysaccharide extracted from seaweed that forms an ionic gel in the presence of divalent cations such as Ca. Easy manipulation of cell density in suspensions of this type of culture offers a well-controlled system for studying cell–cell and cell–matrix interactions. Chondrocytes encapsulated in alginate remain differentiated, as opposed to cells cultured in monolayers, which become fibroblastic (Hauselmann et al., 1994). Moreover, MSCs in beads of this polymer that were cultured in medium containing TGF or BMP-2, -6, or -9 underwent chondrogenic differentiation (Ma et al., 2003; Majumdar et al., 2001). Bovine MSCs seeded in alginate beads also maintained a spherical form and increased the expression of chondrocyte-specific genes under TGF β1 treatment.

In the present trial, the cell–matrix interaction was the priority; therefore, the MSCs were cultured at a low initial seeding density ($1–2 \times 10^6$ cells/mL hydrogel solution), and we attempted to reduce the alginate percentage in the

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collagen hydrogels as much as possible. It was reported that the most prominent MSC chondrogenesis in cells cultured in an alginate layer occurs at an initial density of 25×10^6 cell/mL, while GAG synthesis dropped off precipitously in cultures of lower cell density (Kavalkovich et al., 2002).

Collagen type I is a major organic component of bones. Osteoblasts maintain their phenotype when cultured in collagen type I containing ECM, while MSCs undergo osteogenesis (Lynch et al., 1995; Mizuno et al., 1997). The α2 integrin subunit is a component of a major collagen type I receptor α2β1 integrin. Interaction of this subunit with collagen type I leads to osteoblastic differentiation of MSCs, which is a crucial event in the expression of the osteogenic phenotype (Mizuno et al., 2000). Chondrocytes cultured in collagen type I gel grew in three dimensions, accumulating a cartilaginous ECM while maintaining their round morphology during the culture period (Kimura et al., 1987; Uchio et al., 2002). Furthermore, a cartilage defect was partially repaired when human autologous culture-expanded MSCs engineered in a collagen type I gel were transplanted into an osteoarthritic knee (Wakitani et al., 2002). Our results showed that cells cultured in collagen type I hydrogel and in medium containing TGF β1, and dexamethasone expressed significantly high levels of *coll 1* mRNA. MSCs undergo osteogenic differentiation upon treatment with dexamethasone or TGF β1, which is followed by increased expression of *coll 1* mRNA (Andrades et al., 1999; Gronthos et al., 2003; Phinney et al., 1999). Damaged articular cartilage is usually substituted by fibrous cartilage of which collagen I is a component. We analyzed the influence of collagen type I on MSCs as we were interested in how collagen type I would affect MSC chondrogenesis prior to cell transplantation in a cartilage defect. It appears that collagen type I ECM in combination with TGF β1 and dexamethasone induced both chondrogenesis and osteogenesis, while collagen type II biases towards chondrogenesis. Furthermore, hydrolyzed collagen type II and hydrolyzed collagen type I were able to stimulate collagen type II production in chondrocytes to almost the same extent (Oesser and Seifert, 2003).

Collagen type II is a dominant component of hyaline cartilage. Chondrocytes bind to collagen type II ligands through α1β1, α2β1, and α10β1 integrins resulting in the formation of signaling complexes that play roles in differentiation, matrix remodeling, response to mechanical stimulation, and cell survival (Loeser, 2002). Mitogen-activated protein (MAP) kinase appears to play a central role in mediating the downstream signal from integrins, which can regulate gene expression through activation of transcription factors such as AP-1 and NFκB. Our results clearly demonstrated that the presence of collagen type II in the ECM has the potential to induce and stimulate chondrogenesis. Among all analyzed hydrogels, collagen type II was the most favorable ECM for expressing the chondrogenic phenotype since it resulted in upregulation of each analyzed chondrogenic gene and synthesis of collagen type II and GAG. Upregulation of *sox9*, *coll 2*, *agg, COMP*, and *coll 10* mRNA are believed to represent the physiological adaptation of MSCs to a specialized environment of the articular matrix, which could be partly mediated by collagen type II. Even, results to be most convincing, level of *coll 1* mRNA, which is an indicator of chondrocyte de-differentiation, was the lowest with a tendency of decreasing (Fig 5) (Schnabe et al., 2002). Van der Kraan et al. (2002) found that collagen type II is a better matrix for chondrocytes to express their phenotype than collagen type I. Chondrocytes significantly upregulated DNA and proteoglycan synthesis in a concentration-dependent fashion, according to the presence of collagen type II in the ECM (Scully et al., 2001). In our trial, cell proliferation was also stimulated in collagen-containing hydrogels (data not shown). An earlier investigation that used collagen-GAG matrices found that while the majority of cultured canine chondrocytes seeded in type I collagen had a fibroblastic morphology, the majority of the cells in type II collagen matrices had a chondrocyte morphology and displayed an increase in GAG and collagen type II production (Nehrer et al., 1997; Veilleux et al., 2004).

TGF β1, which is a well-documented potent chondrogenic factor, is stored in a significant concentration in articular cartilage, indicating that it is integral to chondrocyte and matrix homeostasis (Pedrozo et al., 1998). The results from our previous (Bosnakovski et al., 2005) and present studies clearly indicate that MSCs cultured under various culture conditions and treated with TGF β1 undergo chondrogenic differentiation, which is time- and dose-dependent with respect to the stimulants. TGF β1 exerts its effect through a high-affinity interaction with a heteromeric receptor complex (type I and II) that comprises two structurally related serine-threonine kinases. It transduces its signals through the Smad pathway, leading to the activation of the *sox9* transcription mechanisms (Attisano and Wrana, 2002; Hatakeyama et al., 2003). Chondrogenesis (by all parameters) was most prominently induced in the cells cultured in collagen type II ECM and treated with TGF β1. We demonstrated that collagen type II ECM and TGF β1 individually had the potential to induce chondrogenesis and that the best differentiation was achieved when these two factors were combined. The same effect was reported in chondrocytes, and the specific regulation of TGF β1 in the presence of collagen type II was hypothesized to occur through the binding of β1 integrin and its action at the transcriptional, posttranslational, or both levels (Scully et al., 2001). This also confirms that signals received by cells from a soluble regulator such as TGF β1 depend on a combination of factors such as mechanical stimuli, presence of the regulators at the cell differentiation level, and the presence of specific pericellular matrix molecules, for example, collagen type II (Vivien et al., 1990). It was proven by means of stimulating cells cultured in different conditions by exogenous TGF β1 that the mechanism of action of TGF β1 and collagen on chondrocytes is different; however, at the same time, these factors do interact. Cells treated with TGF β1 produce endogenous TGF β1; however, expression of the endogenous product was downregulated in a dose-dependent manner by

the presence of collagen types I or II in the ECM (Qi and Scully, 2002). Shakibaei et al. (1999) reported that the expression of the docking protein Shc, which is associated with the Grb2 adaptor protein and the Ras signal pathway, occurs only when chondrocytes were bound to collagen type II or anti-β1 integrin antibodies. This expression increased with the addition of IGF-I, suggesting collaboration between integrins and growth factors in a common/shared biochemical-signaling pathway. Furthermore, the binding of chondrocyte integrins to ECM molecules, such as fibronectin, results in the intracellular formation of focal adhesion plagues, which is a prerequisite for cells to respond to growth factors such as FGF and IGF-I (Clancy et al., 1997; Martin and Buckwalter, 1998).

In conclusion, to the best of our knowledge, the current study is the first to provide evidence that collagen type II, which acts as a physiological articular cartilage matrix, can initiate and maintain MSC chondrogenesis, and that it enhances the effect of TGF β1. Further understanding of the cell response to ECM will allow more rational designing and development of hydrogels and scaffolds for studying MSC chondrogenic differentiation, articular cartilage development and degeneration, and would advance cartilage tissue engineering.

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