Regulation of Chondrogenesis by Transforming Growth Factor-ß3 and Insulin-like Growth Factor-1 from Human Mesenchymal Umbilical Cord Blood Cells

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ABSTRACT. ***Objective.*** Mature articular cartilage is vulnerable to injuries and disease processes that cause irreversible tissue damage because of its limited capacity for self-repair. Umbilical cord blood is a source of mesenchymal stem cells, which can give rise to cells of different lineages, including cartilage, bone, and fat. Cellular condensation is a required step in the initiation of mesenchymal chondrogenesis. We attempted to differentiate cells from umbilical cord blood into chondrocytes with insulin-like growth factor 1 (IGF-1) and transforming growth factor-ß3 (TGF-ß3).

Methods. Cells were grown in high density micromass and monolayer culture systems and then evaluated for expression of type II collagen, aggrecan, and Sox9. Umbilical cord blood from 130 patients was harvested.

Results. Expression of type II collagen, aggrecan, and Sox9 was detected after 14 days in TGF-ß3- and IGF-1-stimulated cells in both types of culture (monolayer and micromass). On Day 21 in the micromass culture, expression levels were greater than they were at 14 days for all genes. TGF-ß3 was found to be more efficient at promoting chondrogenesis than IGF-1. By western blot, we also found that after 3 weeks, the expression of type II collagen was greater in micromass culture with TGF-ß3.

Conclusion. TGF-ß3 used in micromass culture is the best growth factor for promoting the proliferation and differentiation of mesenchymal cells from umbilical cord blood during chondrogenesis. This approach may provide an alternative to autologous grafting. (First Release May 15 2010; J Rheumatol 2010;37:1519–26; doi:10.3899/jrheum.091169)

Key Indexing Terms:
STEM CELLS OSTEOARTHRITIS CHONDROCYTES COLLAGEN
CELL TRANSPLANTATION CHONDROGENESIS

Although embryonic stem cells are pluripotent, legal and moral controversies concerning their use for therapeutic and clinical applications have led to intense investigation to find a source of progenitor cells in adult organisms. Recently, bone and fat differentiation capacity has been reported for cord blood–derived nonhematopoietic cells[1,2]. In umbilical cord blood (UCB), there exists a primitive stromal population that possesses the characteristics of mesenchymal stromal cells (MSC), as defined by the International Society for Cellular Therapy[3]. It has also been shown that mesenchymal progenitors are present at low frequencies in adult peripheral blood and in UCB[2,4].

Chondrogenesis is a process that is important for the differentiation of chondrocytes during embryogenesis, as well as in adult life (e.g., during skeletal tissue repair). The process begins with the aggregation and condensation of loose mesenchyme. Factors such as transforming growth factor (TGF) are known to play critical roles in the compaction of mesenchymal cells and shaping of the condensations[5]. Insulin-like growth factor 1 (IGF-1) also promotes cell proliferation and matrix synthesis in bone, tendon, muscle, and cartilage cell cultures, and has been shown to enhance aggrecan and type II collagen expression in chondrocyte cultures[6,7]. TGF–ß belongs to a family of proteins that stimulate cell differentiation and extracellular matrix synthesis by a variety of cells, including chondrocytes[8,9].

Precartilage mesenchymal condensation, required for the initiation of chondrogenesis *in vivo*, has been shown to be equally important in *in vitro* cultures[10,11]. Formation of precartilaginous condensations and differentiation of mesenchymal cells into chondrocytes is the beginning of a change in the composition of the extracellular matrix. Due to the crucial role of cellular condensation in the initiation of mesenchymal chondrogenesis[12], in our study the cells were

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cultivated in monolayer and in micromass systems. The micromass culture mimics the cellular condensation phase, increasing the expression of the glycoprotein N-cadherin, which functions as an adhesion molecule during chondrogenesis. This protein has been shown to be essential in this process and is highly expressed in C3H10T1/2 cell cultures in high density cultures (micromass) in the presence of growth factors[13-15]. In monolayer culture, the cells were seeded in the culture plate to reach uniform adherence and to determine if differentiation would occur in the same proportion as that seen in micromass.

The chondrocytes produce cartilage-specific collagen type II[16]. Another gene involved in chondrogenesis is Sox9, a member of the Sox family. The Sox genes comprise a large group of developmentally regulated genes encoding transcription factors that have critical functions in many developmental processes, including skeletogenesis[17]. Sox9 has been shown to have a role in skeletogenesis[18]. During mouse embryonic development, Sox9 and collagen type II, the major structural component and marker of articular cartilage, demonstrate very similar expression patterns, with the expression of Sox9 slightly preceding that of type II collagen[19,20]. Sox9 has been shown to bind to a consensus sequence in the type II collagen enhancer region responsible for chondrocyte-specific expression[21].

Thus, our aim was to isolate mesenchymal stem cells present in UCB and determine their potential for chondrogenic differentiation, measured by expression of collagen type II, aggrecan, and Sox9, in high density (micromass) and monolayer cultures in the presence of IGF-1 and TGF-ß3.

MATERIALS AND METHODS

Isolation and characterization of mesenchymal stem cells. Umbilical blood was collected as described[22], after 39–40 weeks of gestation, from healthy puerperal patients. Written consent was obtained for the procedure. Briefly, blood was collected into a 250-ml standard blood collection bag containing citrate-phosphate-dextrose anticoagulant. The cord blood was processed within 24 h of collection and the mononuclear fraction isolated using standard Ficoll Paque Premium techniques (GE Healthcare, Uppsala, Sweden). The cells were resuspended in modified Eagle's alpha medium (Invitrogen, Carlsbad, CA, USA), with the addition of 10% fetal bovine serum (Gibco BRL, Divinópolis, Brazil), 2 mmol/l L-glutamine (Gibco BRL), and 1% penicillin-streptomycin (Gibco BRL). Cells were then seeded in 25-cm^2 culture flasks (Cellstar) at a density of 1×10^6 mononuclear cells (4×10^5 cell/cm^2) and incubated in a 100% humidified 5% CO_2 atmosphere at 37°C. After 4 days, the cells had adhered to the flask. The supernatant and nonadherent cells were removed and the complete medium was replaced. At 70% confluence, cells were harvested with 0.05% trypsin/EDTA (Gibco BRL) for 5 min at 37°C. Harvested cells were washed twice with phosphate buffered saline (PBS) and 1% fetal bovine serum and replated at a density of 1000 to 2000 cells/cm^2. MSC were obtained from the residual adherent cells.

Flow cytometry analysis. Expression of the following immunophenotypical markers on cultured cells was studied: CD90-PECY5, CD105-PE, CD29-FITC, CD73-PE, STRO-PE, CD34-PE, CD45-SPRD, and HLA-DR-FITC. Monoclonal antibodies specific to each marker were added (20 μl) to the sample (100,000–200,000 cells) and incubated in the dark at room temperature for 25–30 min. Cells were then washed with PBS. Fluorescence was analyzed on a FACScan cytofluorometer using CellQuest software. At least 10,000 cells were analyzed per sample. Forward and side light scatter were measured.

Chondrogenic differentiation. Chondrogenic differentiation of MSC from UCB (passage 2) was performed under serum-free conditions in high density pellet cultures, as described[23,24]. Briefly, the cells were seeded at a density of 5×10^5 cells per 100 μl of medium onto dry wells in a 96-well plate (Corning Life®). After 2 h, the wells were slowly flooded with 0.2 ml of chondrogenic medium. A second model of culture was a monolayer system, which was seeded in Costar 6-well cell culture plates (Corning) at a density of 1×10^6 cells/cm^2. Chondrogenesis was induced by adding 10 ng/ml TGF-ß3 (R&D Systems, Minneapolis, MN, USA) or 100 ng/ml recombinant human IGF-1 (R&D Systems). Cells were collected at 7, 14, and 21 days after induction (Figure 1).

RNA isolation and real-time RT-PCR. After 7, 14, and 21 days of growth after induction, total RNA was isolated from the pellets or cells cultured in monolayer and the control group (MSC from UCB). Total mRNA was prepared using Trizol reagent according to the manufacturer's instructions (Invitrogen). One microgram of total RNA was treated with 1 U of deoxyribonuclease I (DNase I; Invitrogen) to digest genomic DNA. Reverse transcription was performed with SuperScript II (Invitrogen) according to the manufacturer's protocol. Quantitative real-time polymerase chain reaction (PCR) was performed on a 96-well/plate ABI Prism 7000 Sequence Detection machine (Applied Biosystems, Foster City, CA, USA) using SyberGreen PCR Master Mix (Applied Biosystems). The total volume (12 μl) of each PCR reaction contained 6 μl SybrGreen PCR Master Mix, 10 ng cDNA (3 μl), and 150 pM (3 μl) of each of the forward and reverse primers. Real-time PCR was carried out at 95°C for 10 min (activation), 40 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 20 s (amplification), and 72°C for 1 min final extension. The melting curves were acquired after PCR to confirm the specificity of the amplified products. A standard curve based on cycle threshold values was used to evaluate gene expression. GAPDH was used as an internal control.

Primer sequences. Primer sequences for all genes were designed using the ABI Primer Express program (Applied Biosystems). The specific primers used for type II collagen (Col II), aggrecan, Sox9, and GAPDH were as shown in Table 1.

Western blot analysis. To analyze proteins, the proteins secreted by the cells in culture medium were precipitated using 2 mg/ml of pepsin (Sigma-Aldrich) and 30 μl/ml of glacial acetic acid. The samples were incubated at 30°C for 30 min and stored at 4°C overnight, under shaking. Then the samples were centrifuged for 90 min at 5000 g. Finally, the pellet was washed twice with PBS buffer, and the quantity of protein obtained was determined by spectrophotometer. The 30 μg of proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose Hybond membranes (Amersham) using a transblot apparatus (Invitrogen). The membranes were blocked with 5% (vol/vol) skim milk in PBS containing 0.1% Tween 20 and then incubated with mouse anti-rabbit type II collagen antibody (Chemicon) diluted 1:2000. Membranes were rinsed 3 times with PBS containing 0.1% Tween 20, and then incubated with a peroxidase-conjugated anti-rabbit secondary antibody. Signals were visualized using an ECL kit (Amersham).

Statistical analysis. Statistical analysis was performed by one-way analysis of variance (ANOVA) using commercial software (InStat; GraphPad Software, San Diego, CA, USA; http://www.graphpad.com). The confidence interval was 95%. Taking into account the multiple comparisons between groups, p values < 0.05 were considered significant.

RESULTS

Cells firmly attached to the surface of the cell culture plate presented a spindle-shaped, fibroblast-like morphology, were agranular, and formed colonies. Subcultured cells grown in a monolayer maintained a stable fibroblast-like morphology with no signs of granulation.

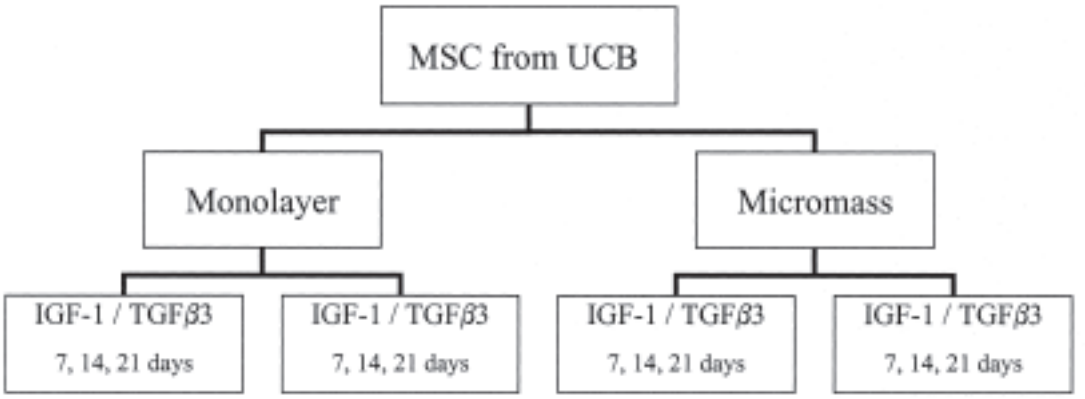

Figure 1. Cells were collected at 7, 14, and 21 days after induction of chondrogenesis. MSC: mesenchymal stromal cells; UCB: umbilical cord blood.

Table 1. Specific primer sequences used in quantitative RT-PCR for type II collagen (Col II), aggrecan, Sox9, and GAPDH.

		Primer
Type II collagen	Sense	GGC AAT AGC AGG TTC ACG TAC A
	Antisense	CGA TAA CAG TCT TGC CCC ACT T
Aggrecan	Sense	TCG AGG ACA GCG AGG CC
	Antisense	TCG AGG GTG TAG CGT GTA GAG A
Sox-9	Sense	CGA CCG TCA AGG CTG AGA AC
	Antisense	CCA CTT GAT TTT GGA GGG ATC T
GAPDH	Sense	GAC TTC CGC GAC GTG GAC
	Antisense	GTT GGG CGG CAG GTA CTG

Immunophenotypic characterization of mesenchymal cells. Immunophenotypic characterization of the mesenchymal umbilical cord blood cells was carried out. The percentages of positive cells for the cell-surface markers analyzed by flow cytometry were as follows: CD90-PE 95.36%, CD105-PE 95.35%, CD29-FITC 92.28%, CD73 90.20%, STRO1-PE 65.29%, CD34-PE 0.15%, CD45-SPRD 0.41%, and HLA-DR-FITC 0.03%. This profile is consistent with a nonhematopoietic cell and confirmed that hematopoietic cells had been depleted from the cultures (Figure 2).

Induction of cartilage-specific gene expression in UCB mesenchymal cell cultures by TGF-ß3 or IGF-1. Type II collagen, aggrecan, Sox9, and GAPDH expression were investigated by real-time PCR, following normalization of the gene levels of each in monolayer and micromass cultured cells stimulated with TGF-ß3 or IGF-1 for 7, 14, and 21 days. In both groups (monolayer and micromass) there was no expression of type II collagen, aggrecan, and Sox9 after 7 days of induction.

Type II collagen. All groups of cells stimulated with TGF-ß3 in both systems of culture showed expression of type II collagen after 14 days.

TGF-ß3: In the monolayer system, expression levels were greater on Day 14 than those in the micromass system ($p < 0.001$). Monolayer culture cells stimulated with TGF-ß3 showed expression of type II collagen at 14 days postinduction, but this expression decreased at 21 days ($p < 0.001$). However, there was no statistically significant difference between micromass cultures stimulated with TGF-ß3 on Days 14 and 21. On Day 21, expression levels were not significantly different between the 2 systems (Figure 3A).

IGF-1: Type II collagen expression in cells stimulated with IGF-1 in both culture systems was time-dependent: on Day 21, expression levels were greater than on Day 14 in micromass ($p < 0.001$) and in monolayer ($p < 0.01$). Expression of type II collagen was greater on Day 14 in micromass culture than on Day 21 in the monolayer culture ($p < 0.05$). There was a statistically significant difference on Day 21 between cells kept in monolayer and those in micromass culture ($p < 0.001$; Figure 3B).

Aggrecan. All groups of cells stimulated with TGF-ß3 showed expression of aggrecan on Day 14 in both culture systems.

TGF-ß3: On Day 14, expression levels in the micromass culture were greater than on Day 21 in the monolayer system ($p < 0.001$). In addition, expression levels were greater on Day 14 in the micromass culture than in the monolayer culture on the same day ($p < 0.01$). There was no statistically significant difference between the systems on Day 21 (Figure 3C).

IGF-1: The expression of aggrecan in the monolayer culture was lower on Day 14 than on Day 21 ($p < 0.001$). This same time-dependent relationship was seen in the micromass system ($p < 0.001$). For both culture systems, levels of expression on Day 14 were not statistically different. On Day 21, expression levels were greater in the micromass than in the monolayer system ($p < 0.001$; Figure 3D).

SOX9. After stimulation with TGF-ß3 or IGF-1, the 2 culture systems showed Sox9 expression after 14 days.

TGF-ß3: There was no statistically significant difference between cells kept in monolayer and micromass culture on Day 14. The cells in monolayer showed greater levels of

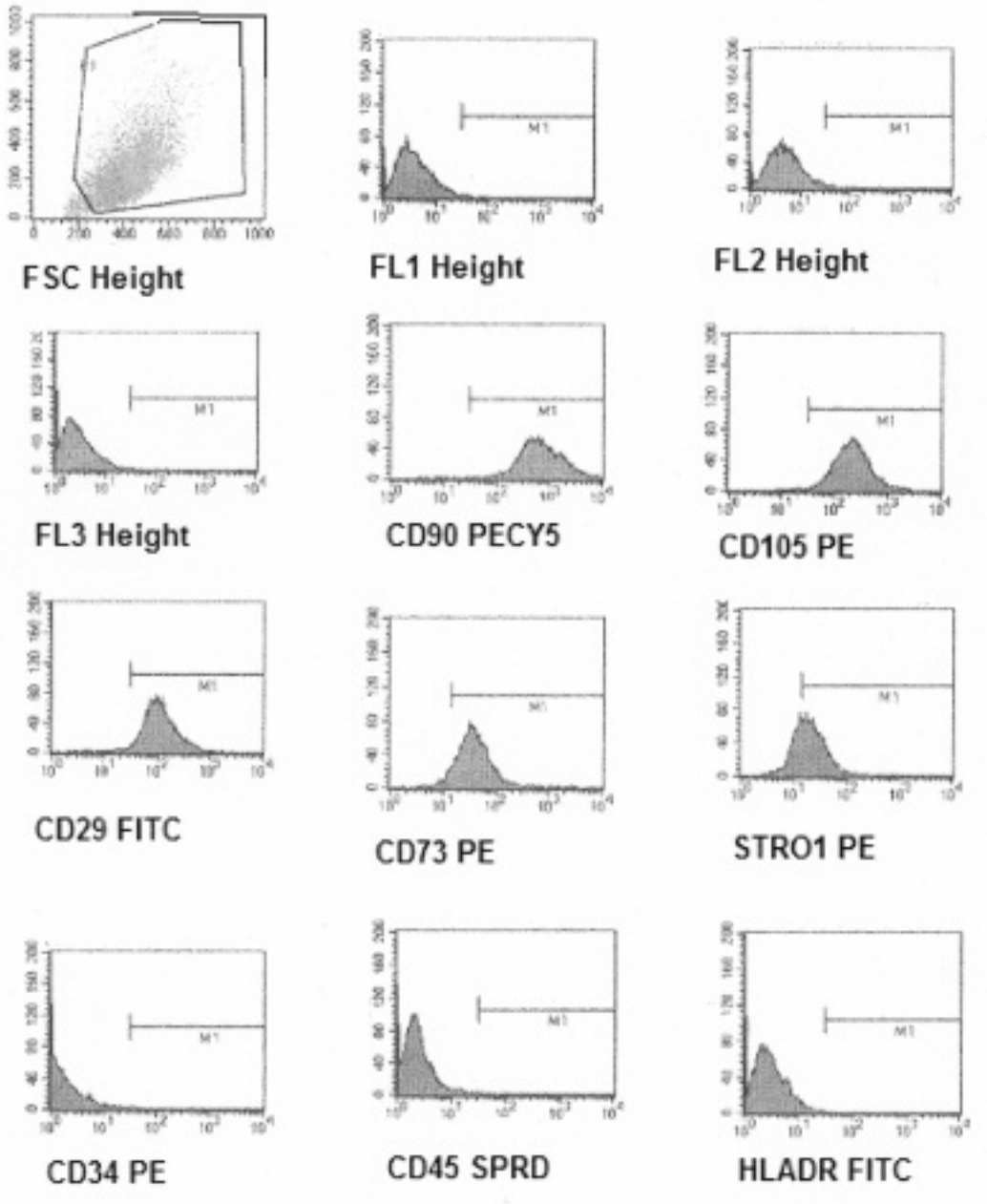

Figure 2. Percentages of positive cells for the cell-surface markers analyzed by flow cytometry were as follows: CD90-PE 95.36%, CD105-PE 95.35%, CD29-FITC 92.28%, CD73 90.20%, STRO1-PE 65.29%, CD34-PE 0.15%, CD45-SPRD 0.41%, and HLA-DR-FITC 0.03%. This profile is consistent with a nonhematopoietic cell and confirmed that hematopoietic cells had been depleted from the cultures.

expression on Day 14 than those in micromass culture on Day 21. In the micromass system, expression levels on Day 14 were greater than those in the monolayer culture on Day 21 ($p < 0.001$). In addition, for the micromass cultures, expression levels on Day 14 were greater than on Day 21, and expression levels in the monolayer culture on Day 21 were lower than those in the micromass culture on the same day (Figure 3E).

IGF-1: For cells stimulated with IGF-1, Sox9 expression on Day 14 in both culture systems was lower than on Day 21 ($p < 0.001$). However, levels of expression in the micromass culture on Day 21 were greater than those in the monolayer culture on the same day ($p < 0.001$; Figure 3F).

Induction of type II collagen protein expression in UCB mesenchymal cell cultures by TGF-ß3 or IGF-1. Production of type II collagen in the culture medium was detected in both culture systems (monolayer and micromass) with both growth factors (IGF-1 and TGF-ß3; Figure 4). The proteins

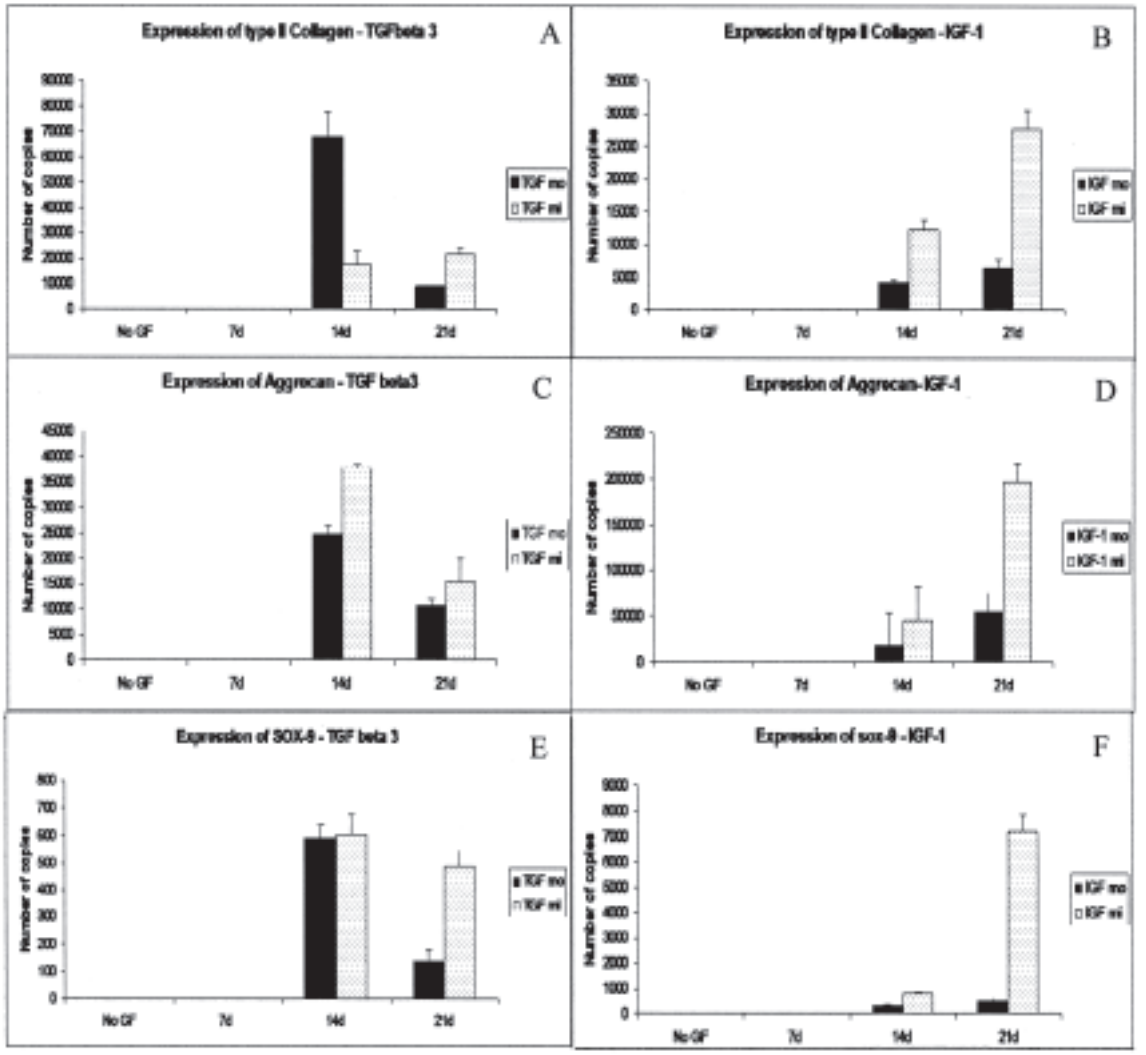

Figure 3. Gene expression of type II collagen, aggrecan, and Sox-9. A. In the monolayer system, expression levels were greater Day 14 than those in the micromass system ($p < 0.001$). Monolayer culture cells stimulated with TGF-ß3 showed expression of type II collagen at 14 days postinduction, but this decreased at 21 days ($p < 0.001$). There was no statistically significant difference between micromass cultures stimulated with TGF-ß3 on Days 14 and 21. On Day 21, levels were not significantly different between the 2 systems. B. Type II collagen expression in cells stimulated with IGF-1 in both culture systems was time-dependent: on Day 21, levels were greater than on Day 14 in micromass ($p < 0.001$) and in monolayer culture ($p < 0.01$). Expression of type II collagen was greater on Day 14 in micromass culture than on Day 21 in the monolayer culture ($p < 0.05$). There was a statistically significant difference on Day 21 between cells kept in monolayer and micromass cultures ($p < 0.001$). C. On Day 14, levels in the micromass culture were greater than on Day 21 in the monolayer system ($p < 0.001$). In addition, levels were greater on Day 14 in the micromass culture than in the monolayer culture on the same day ($p < 0.01$). There was no statistically significant difference between the systems on Day 21. D. Expression of aggrecan in the monolayer culture was lower on Day 14 than on Day 21 ($p < 0.001$). This same time-dependent relationship was seen in the micromass system ($p < 0.001$). For both culture systems, levels on day 14 were not statistically different. On Day 21, levels were greater in the micromass than in the monolayer system ($p < 0.001$). E. There was no statistically significant difference between cells kept in monolayer and micromass cultures on Day 14. Cells in monolayer showed greater levels of expression on Day 14 than those in micromass culture on Day 21. In the micromass system, levels on Day 14 were greater than those in monolayer culture on Day 21 ($p < 0.001$). In addition, for the micromass cultures, levels on Day 14 were greater than on Day 21; levels in the monolayer culture on Day 21 were lower than those in the micromass culture on the same day. F. For cells stimulated with IGF-1, Sox9 expression on Day 14 in both culture systems was lower than on Day 21 ($p < 0.001$). However, levels in the micromass culture on Day 21 were greater than those in the monolayer culture on the same day ($p < 0.001$).

secreted by the cells in culture medium were precipitated. Purity of the isolated type II collagen was confirmed by SDS–PAGE and Western blotting with anti-human type II collagen antibody.

DISCUSSION

For regeneration of articular cartilage using cell-based therapies, it is necessary to identify the available cells that have the ability to differentiate into chondrogenic cells. MSC

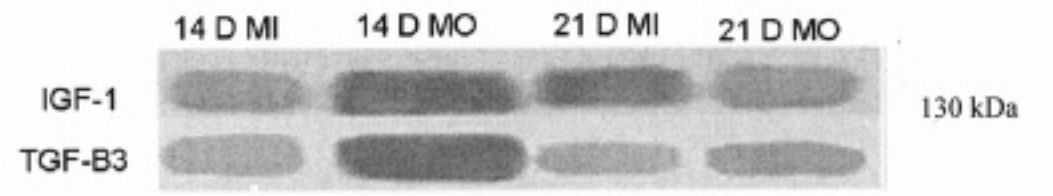

Figure 4. Western blotting cell stimulated with TGFß3 and IGF-1. Proteins secreted by cells in culture medium were precipitated. Purity of isolated type II collagen was confirmed by SDS-PAGE and Western blotting with anti-human type II collagen antibody.

from UCB are a new potential stem cell source that has been shown to have high self-renewal capabilities and pluripotency[25]. We demonstrated that these cells can differentiate toward a chondrogenic lineage, thus establishing the viability of using these cells for cartilage regeneration. We found that both TGF-ß3 and IGF-1 were effective in promoting expression of type II collagen, aggrecan, and the transcription factor Sox9 by the MSC from UCB after 14 days in culture. This result is in agreement with previous studies that promoted chondrogenic differentiation in MSC from other sources[26,27]. Expression of the transcription factor Sox9 preceded expression of type II collagen, which is expressed exclusively by articular chondrocytes[21]. When Sox9 is not expressed, the chondrocytes become hypertrophied and produce a calcified cartilage[28].

The earliest event of embryonic limb development is cellular condensation, whereby precartilage mesenchymal cells aggregate as a result of specific cell-cell interactions, a requisite step in the chondrogenic pathway[29]. Thus, cellular condensation has been described as pivotal to chondrogenic differentiation. Nevertheless, we found that MSC from UCB stimulated with TGF-ß3 were able after 14 days to express proteins of the articular matrix, including type II collagen, aggrecan, and Sox9, even in a monolayer culture system. However, this expression diminished on Day 21 in the monolayer, likely because the ECM changes in chondrocytes kept in a monolayer, and these changes are known to cause dedifferentiation[30]. Expression of type II collagen by cells cultured in the micromass system with TGF-ß3 was lower than in the monolayer cultures on Day 14; however, this expression remained steady on Day 21, suggesting that the culture system can maintain the differentiated phenotype for longer in *in vitro* culture.

TGF-ß3 has been shown to be a potent inducer of chondrogenesis in a variety of cell types[31-35], and even in monolayer culture[36,37]. However, the greater challenge is to maintain the chondrocyte-like phenotype and avoid dedifferentiation.

Expression of aggrecan could be observed in both culture systems, as the cells were stimulated with TGF-ß3. This macromolecule is present not only in articular chondrocytes but also hypertrophic chondrocytes and growth plate[38]. In addition, the expression of aggrecan is regulated by other mechanisms that do not depend only on adhesion molecules. However, the expression of this macromolecule was greater on Day 21 in the micromass system. These findings are in agreement with another report[39].

Following stimulation with IGF-1, we observed a time-dependent relationship: on Day 21, expression of type II collagen was greater than on Day 14 in both systems, although the increase was lower in the monolayer than in the micromass system. IGF-I is an anabolic agent that regulates chondrocyte proliferation and biosynthesis at all stages of cartilage development[40,41]. Moreover, IGF-I promotes collagen synthesis and/or inhibits collagen breakdown in the collagen metabolism process[42]. In contrast with the transitory effect of TGF-ß3, the time-dependent chondrogenic response of these cells to IGF-1 might occur because of an inhibitory effect on the collagen breakdown, in contrast to what was observed with TGF-ß3. In contrast to collagen in the expression level, there was a lower increase in the monolayer than in the micromass culture.

In addition, the cells in micromass culture produced more type II collagen on Day 14 than those in monolayer on Day 21, suggesting that the micromass system is more efficient. Tuli, *et al*[43] showed that MSC require the precise control of N-cadherin expression, dependent on TGF-ß3-initiated MAP kinase signaling cascades, most likely to mediate the appropriate cell-cell adhesion required for precartilage mesenchymal condensation and the ensuing differentiation. The micromass system enhances the production of N-cadherin.

In addition to the expression of these genes in the neoformed cells, another objective of our study was to evaluate the production of these proteins using immunoblotting. Type II collagen is a protein that the cell produces and secretes to the extracellular matrix. We investigated the presence of this protein in culture medium and confirmed that the cells were producing type II collagen. Therefore we confirmed that the transcripts were translated into protein.

Moreover, we found that the Sox9 transcription factor was expressed at higher levels in monolayer cultures on Day 14 than on Day 21. This is the same pattern seen for type II collagen production, reinforcing that Sox9 is a transcription factor associated with the induction of chondrogenesis of articular chondrocytes and type II collagen expression, as shown previously[28].

While it has been demonstrated that TGF-ß3 is a potent inducer of type II collagen expression[8], other studies also found that stimulation with IGF-1 resulted in the largest

increase in type II collagen expression[44,45]. Moreover, IGF-1 has been reported to have effects on the mitogenic activity and matrix synthesis of articular and growth plate chondrocytes, which is why expression of Sox9 was lower in cells stimulated with this growth factor[44,45]. While we found that TGF-ß3 showed better induction efficiency in monolayer cultures after 14 days, it maintained type II collagen expression for longer in the micromass culture. Some groups have used TGF-ß3 in association with IGF-1 to promote chondrogenic differentiation[46,47]. There are also controversial reports that treatment with TGF-ß superfamily members is a key requirement for the *in vitro* chondrogenic differentiation of MPC[43]. By studying the action of both TGF-ß3 and IGF-1, we found both are capable of differentiating UCB-derived MSC to chondrocytes after 14 and 21 days in culture. However, after 21 days of induction, TGF-ß3 was more efficient in maintaining the chondrocyte phenotype in the micromass culture system; in contrast, expression of the Sox9 transcription factor diminished on Day 21 in the monolayer system, suggesting the initiation of dedifferentiation. In the micromass culture, expression of both collagen and Sox9 remained level. Thus, we conclude that UCB-derived MSC are best differentiated for 21 days in the micromass system. We also conclude that UCB-derived MSC can effectively undergo chondrogenesis to produce articular chondrocytes.

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