Comparison of Phenotypic Characterization between “Alginate Bead” and “Pellet” Culture Systems as Chondrogenic Differentiation Models for Human Mesenchymal Stem Cells

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Chondrogenesis involves the recruitment of mesenchymal cells to differentiate into chondroblasts, and also the cells must synthesize a cartilage-specific extracellular matrix. There were two representative culture systems that promoted the chondrogenic differentiation of human mesenchymal stem cells. These systems were adaptations of the “pellet” culture system, which was originally described as a method for preventing the phenotypic modulation of chondrocytes, and the “alginate bead” culture system, which was used to maintain encapsulated cells at their differentiated phenotype over time, and also it was used to maintain the cells’ proteoglycan synthesis at a rate similar to that of primary chondrocytes. We performed the differences on the phenotypic characterization with the two methods of differentiating human mesenchymal stem cells into chondrocytes. The typical gene for articular cartilage, collagen type II, was more strongly expressed in the “alginate bead” system than in the “pellet” culture system, in addition, specific gene for hypertrophic cartilage, collagen type X, was more rapidly expressed in the “pellet” system than in “alginate bead” culture system. Therefore, the “alginate bead” culture system is a more phenotypic, practical and appropriate system to differentiate human mesenchymal stem cells into articular chondrocytes than the “pellet” culture system.

Key Words: Human mesenchymal stem cell, alginate bead, pellet, chondrogenesis, articular cartilage

INTRODUCTION

Bone marrow stromal cells (BMSCs) exist as a population of cells having the capacity to differentiate into osteogenic, chondrogenic or adipogenic lineages both in vitro and when implanted subcutaneously in SCID mice.1 These cells may be isolated from the bone marrow using standardized techniques and then they are expanded in culture; they maintain their capacity to differentiate along these aforementioned pathways when they are subjected to appropriate culture conditions.2,3 This property of mesenchymal stem cells creates therapeutic opportunities for the treatment of damages in mesenchymal tissues.4,5,6 To improve the repair of bone and cartilage, several modalities such as bone marrow alone, bone marrow-scaffold composites, and culture expanded MSC implantation have been tried.5,6,7

Two representative culture systems have been developed that promote the chondrogenic differentiation of human marrow mesenchymal progenitor cells. These systems are adaptations of the “pellet” culture system that was originally described as a method for preventing the phenotypic modulation of chondrocytes, and the “carriers” culture system including collagen gels, polylactic acid, polyglycolic acid, agarose, and alginate for maintaining the encapsulated cells as their differentiated phenotype over time.3,4,5,6 Among these carriers, alginate induced the chondrogenic phenotype in MSCs in vitro, and this substance had the necessary physical characteristics and handling properties to support the cells and also serve...
as a carrier to fill full-thickness osteochondral defects in vivo. In addition, freshly isolated articular chondrocytes cultured in "alginate beads" for 2 weeks synthesized a matrix similar to that of native articular cartilage: these cells maintained their phenotype for as long as eight months, and it has been demonstrated that culturing of de-differentiated chondrocytes in alginate induces re-expression of the differentiated chondrocyte phenotype. The "pellet" culture system involves formation of cell aggregates by a simple one-step centrifugation method. Moreover, this system has more recently been used in studies of the terminal differentiation of growth-plate chondrocytes. This culture system allows cell to cell interactions analogous to those that occur in precartilage condensation during embryonic development.

However, these cell configurations are not yet sufficient for the induction of chondrogenesis: the chondrogenic differentiation from the marrow-derived progenitor cells required the use of a defined medium to which was added certain bioactive factors including insulin, transferrin, selenium, pyruvate and TGF-β. Members of the transforming growth factor-β (TGF-β) family of growth factors have been shown to play major roles for bone and cartilage development. TGF-β evokes a chondrogenic response in embryonic mesenchymal cells, and periosteum-derived cells. Several studies have shown that TGF-β isoforms differ in their effects on the chondroprogenitor cells and other cells. TGF-β can induce chondrogenic differentiation from mouse or human bone marrow stromal cells, as has been judged by the expression of aggrecan and type II collagen, IX collagen and so on. These systems have provided the means for studying the process of chondrogenesis, including studying those factors that regulate the progression of cells through the entire chondrogenic lineage.

One of the most important parts of chondrogenesis from human mesenchymal stem cells is being able to find a reasonable and the most ideal differentiation method for clinical applications. The terminal differentiation, as judged by the expression of type X collagen, may have the possibility of bone formation, as has been observed in the growth plate or during the process of osteoarthritis. Yet there is not much information to explain the correlation between type II and type X collagen expression. Therefore, we need to compare not only type II collagen, but also type X collagen expression when using the two methods.

In this work, we have studied the phenotypic characterization of TGF-β-induced chondrogenesis of human mesenchymal stem cells; these cells were isolated from bone marrow through two representative chondrogenic differentiation processes, and they were grown in two popular culture systems: the "pellet" culture system and "alginate bead" culture system.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium containing low glucose (DMEM-LG, 1g/L D-glucose, L-glutamine, pyridoxine hydrochloride and 110mg/L sodium pyruvate), Dulbecco's modified Eagle's medium containing high glucose (DMEM-HG, 4.5g/L D-glucose, L-glutamine, pyridoxine hydrochloride and 110mg/L sodium pyruvate), trypsin-EDTA, antibiotic-antimycotic solution (penicillin, streptomycin, and fungizone) and insulin-transferrin-selenoide (ITS) were all purchased from Gibco BRL (Gibco BRL, Grand Island, NY, USA). Oligo (dT), dNTP mix, 5X first strand buffer, RNase inhibitor, 10X polymerase chain reaction (PCR) buffer, Taq DNA polymerase and the total RNA isolation kits were purchased from Qiagen (Qiagen, Valencia, CA, USA). Alginic acid, ascorbic acid, Safranin O and Alcian blue were obtained from Sigma Chemical Co. (Sigma, St. Louis, MO, USA). Falcon plastic ware including the 6-well culture plates, 15 mL polypropylene tubes and 75cm² flasks were procured from Becton-Dickinson Labware (Franklin Lakes, IN, USA). Transforming growth factor β3 was purchased from R&D (R&D Systems, Minneapolis, MN, USA).

Methods

Culture of mesenchymal stem cells

A syringe containing 3,000 units of heparin was
used to aspirate 2–8 mL of bone marrow from the posterior iliac crest of healthy adult donors. Our study was approved by the Institutional Review Board of our local hospital, and an informed consent was obtained from all cell donors. DMEM-LG was added to the aspirate, and a cell pellet was produced by centrifugation to eliminate the fatty marrow. The pellet was next fractionated on a density gradient generated by mixing and then centrifuging the pellet in successive 25, 50 and 75% Percoll solutions (Amersham Pharmacia, Piscataway, NJ, USA) at 13,000 rpm for 20 minutes each. The middle of 50% of the gradient was collected. The medium for the human mesenchymal stem cells consisted of DMEM-LG supplemented with 10% FBS and 1% antibiotic-antimycotic solution. The bone marrow stem cells were selected based on their ability to adhere to the tissue culture plastic; nonadherent hematopoietic cells were removed with the culture medium when the medium was replaced after 3 days of culture. The medium was changed twice per week thereafter. When the primary bone marrow stem cells became nearly confluent after approximately 1–2 weeks, we detached them by using 0.25% trypsin-1 mM ethylenediaminetetraacetic acid (EDTA). The cells were replated until they had experienced 2-3 passages in a monolayer culture, and they were then differentiated in a 3-dimensional culture.

Characterization of mesenchymal stem cells
At 21 days of culture, the cells were harvested from the tissue culture flasks by a treatment of 5 mM EDTA in phosphate-buffered saline (PBS). The cells were in a solution at a concentration of $5 \times 10^6$ cells/mL, and they were stained for 45 min with an empirically determined amount of each antibody: the MSC-specific marker CD105 (Ancell Corp., Bayport, MN, USA), the hyaluronic acid receptor CD44 (Santa Cruz Biotech, Santa Cruz, CA, USA), β1 integrin CD29 (Chemicon International, Temecula, CA, USA), the early hematopoietic progenitor cell marker CD34, the monocyte/macrophage marker CD14, or the leukocyte common antigen CD45 (BD Bioscience, Franklin Lakes, NJ, USA). The labeled cells were thoroughly washed with two volumes of PBS and then they were fixed in a flow buffer (1% paraformaldehyde, 0.1% sodium azide and 0.5% bovine serum albumin in PBS). Nonspecific fluorescence was determined by using equal aliquots of the cell preparation that were incubated with anti-mouse monoclonal antibodies (PharMingen). The labeled cells were analyzed on a FACScan (Becton Dickinson Instrument, San Jose, CA, USA) by collecting 10,000 events with the Cell-Quest software program (Becton-Dickinson).

Chondrogenic differentiation in two systems
There are two representative systems for the chondrogenic differentiation of human mesenchymal stem cells: the “Pellet” culture system and “alginate bead” culture system.

"Pellet" culture
The chondrogenic potential was tested in a defined medium that has been shown to promote chondrogenesis in human marrow cells. To initiate these cultures, $1 \times 10^6$ freshly trypsinized cells were placed into 15 mL polystyrene tubes containing 0.5 mL of a DMEM-HG, and then they were centrifuged for 5 min at 1,200 rpm. The cells were then cultured in DMEM-HG plus 1X ITS-A, 10 ng/mL TGF-β3 and 50 μg/mL ascorbic acid. The caps of the tubes were loosened to permit gas exchange, and the tubes were maintained at 37°C in 5% CO₂, 95% air. Within 24 hr of incubation, the cells formed an essentially spherical aggregate that did not adhere to the walls of the tube. Changes of the medium were carried out at 3 day intervals and the aggregates were harvested at time points up to 14 days.

"Alginate bead" culture
Alginate beads that contained $2 \times 10^6$ cells/mL were prepared in the proportion of 3 mL of cell suspension mixed with 3 mL of sterile low-viscosity alginate gel (2.4%, cell prep alginate). The suspension was then transferred into a 10 cc sterile syringe and expressed through a 19-gauge needle. The suspended mesenchymal stem cells were slowly expressed in a drop-wise fashion, and the gelation of the alginate beads occurred instantaneously into 5 mL of 102 mM CaCl₂ in 6-well plates (20 beads/well, with each bead containing an average of $5 \times 10^6$ cells). The average bead diameter was 2.5 mm, and this was established.
with a linear micrometer that was used as a reticle. After instantaneous gelation, the beads were allowed to further polymerize for a period of 15 min in CaCl₂ solution. The beads were washed three times with 0.15 M NaCl and then washed once with DMEM-HG. The beads were cultured for 3, 5, 7 and 14 days in 5 mL of a defined medium (DMEM supplemented with ITS-A) that was also supplemented with 10 ng/mL of TGF-β3 and 50 μg/mL ascorbic acid. The culture media was changed every 3 day. After the defined period of days, the alginate beads were washed three times with PBS and next dissolved for 15 min with a chelating agent, 40 mM ethylenediaminetetraacetic acid (EDTA). Then, the cell suspension was centrifuged at 2,000 g for 5 min and the cell pellets were stored at -20°C.

**RNA preparation and analysis**

For evaluating the changes of each gene expression, semi-quantitative reverse-transcription polymerase chain reactions (RT-PCR) were done. The total RNA was isolated from the cell pellets by the RNeasy kit (Qiagen). The isolated RNA was quantitated by the spectrophotometer. The primer sets used for the aggrecan, collagen type I, II and X, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, a housekeeping gene) were derived from sequences available through Gen Bank. The sequences used for each set are detailed in Table 1. One microgram of total RNA was reverse-transcribed with the use of the Omniscript kit (Qiagen). Aliquots (2 μl) of the resulting chain reaction buffer, 0.2 μM of the dNucleoside triphosphate mixture, 0.5 μM of each primer, and 1 U of Taq DNA polymerase (Qiagen). The identity of each PCR product was confirmed by sequence analysis. For all the reported experiments, the conditions were determined to be in the linear range for both the polymerase chain reaction amplification and the image analysis system. The 2.0 μL aliquots of the 50 μL reverse-transcription sample were subjected to polymerase chain reaction with the number of cycles ranging from 25 to 40 cycles. Each of the polymerase chain reaction products was analyzed by electrophoresis on a 1.5% agarose gel containing 0.1 μg/μL ethidium bromide (EtBr). The photographs were obtained and quantitated with use of a densitometer. The density values for the genes were then normalized to GAPDH values to yield a semi-quantitative assessment. Normalization to GAPDH, in addition to serving as a housekeeping gene for semi-quantitative assessment, also controled for differences in efficiency between the reverse transcription and the polymerase chain reaction.

**Histological evaluation**

Alginate beads and pellets were harvested and fixed in 10% buffered formaldehyde at room temperature; they were rinsed with PBS, serially dehydrated, infiltrated with aryl acetate, paraffin embedded and sectioned at 5-μm thickness for hematoxylin-eosin (HE) stains, and histochemical and immunohistochemistry evaluation. Histochemical stains were performed according to standardized protocols for alcian blue and safranin O stain. The sections were rinsed with PBS, and then they were stained overnight with 1% alcian blue dye at pH 1.0 and 0.1% aqueous safranin-O for 10

**Table 1. Oligonucleotide Primers for PCR Amplification of Probe and Transcript Sizes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Length (mer)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggrecan</td>
<td>5'- GAATCTAGAGCTGAGACGTC - 3'</td>
<td>20</td>
<td>540</td>
</tr>
<tr>
<td></td>
<td>5'- CTGCAGCTTTATCGAT - 3'</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Collagen I</td>
<td>5'- TCCGACCCTCTCTCTGTA - 3'</td>
<td>20</td>
<td>295</td>
</tr>
<tr>
<td></td>
<td>5'- GAGTGGGGTTATGGAGG - 3'</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Collagen II</td>
<td>5'- TCCGACCCTCTCTCTGGA - 3'</td>
<td>20</td>
<td>368</td>
</tr>
<tr>
<td></td>
<td>5'- GAGTGGGGTTATGGAGG - 3'</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Collagen X</td>
<td>5'- CCCAAGAGGTGCCCCCTGAATAC - 3'</td>
<td>24</td>
<td>703</td>
</tr>
<tr>
<td></td>
<td>5'- CCTGAGAAAGAGGTGGACATA - 3'</td>
<td>24</td>
<td></td>
</tr>
</tbody>
</table>
min at room temperature to detect the sulfated proteoglycan matrix.

Evidence of chondrogenesis was assessed based on immunohistochemical (IHC) detection of the primary matrix proteins of articular cartilage, including collagen type II. IHC stains were performed on the paraffin-embedded tissues with an antibody to the following antigen (collagen II (COL II): 1:500; ICN Biomedicals Inc., Ohio, USA). Briefly, after deparaffinization, blocking of endogeneous peroxidase was done with 3% H₂O₂ for 15 minutes. Sections were then placed in BSA for 10 minutes at room temperature. The primary antibodies were applied and allowed to react for overnight at 4°C. Sections were then incubated with a secondary biotinylated anti-mouse/anti-goat antibody and peroxidase labeled streptavidin (LSAB2 System, DAKO). After color development using Histostain-SP with AEC (Dako), the coverslips were applied and the slides were examined using conventional light microscopy. Each positively stained experimental sample was then quantified using the image analyzer (MetaMorph, Universal Imaging Corp., PA, USA) by three observers. For the alcian blue and safranin O staining, quantification of positive stained section at 14 days were normalized to that observed at 1 day because the alginate matrix and matrix that the cells produced could not be distinguished by in situ histology.¹⁶

Statistical analysis
The image analysis for histological evaluation was repeated three times. Experimental values were expressed as percentage of the positive area. Comparisons between the two groups were analyzed by student’s t-test. The data were expressed as the mean ± standard deviation (S.D.). Values of p<0.05 were considered to be statistically significant.

RESULTS
Characterization of human mesenchymal stem cells
Flow cytometric analysis of undifferentiated human MSCs was done. The histograms are for a series of 6 different monoclonal antibodies incubated with MSC suspensions. The thick lines in Fig. 1 represent the specific antibody fluorescence, whereas the thin lines represent fluorescence from the nonspecific isotype control antibodies. Significant and uniform shifts in fluorescence were observed for the samples incubated with the antibody, while the markers CD14, CD34 and CD45, which were diagnostic of hematopoietic cells, were completely negative in MSC cultures. The histograms were representative of those obtained from at least five different MSC donors.

Chondrogenic differentiation of human mesenchymal stem cells

RT-PCR
In order to compare the temporal patterns of expression of these matrix components during

Fig. 1. FACS analysis of human mesenchymal stem cells. The cells were harvested and labeled with antibodies against CD14, CD29, CD34, CD44, CD45 and CD105 or they were treated with control IgGs, as indicated and analyzed by FACS. The plots show the isotype control IgG-staining profiles (thin lines) versus the specific antibody staining profile (thick lines). Significant and uniform shifts in fluorescence were observed in the samples incubated with the antibodies such as CD105 (97.64%), CD44 (57.19%), and CD29 (98.9%), while the markers that were diagnostic of hematopoietic cells, CD14 (4.3%), CD34 (8.46%), and CD45 (0.38%), were negative in the MSC cultures.
differentiation of chondrocyte between the pellets and alginate beads, we were analyzed the gene expression by RT-PCR. The identity of each targeted PCR amplification product was confirmed by DNA sequence analysis of the agarose gel-purified bands to exclude the possibility of false positive results (Fig. 2). Aggrecan mRNA was rapidly upregulated within 3 day in the “alginate bead” culture; however, it appeared somewhat more slowly in the “pellet” culture, and it reached consistent levels of expression within 7-14 days. The type I collagen gene was uniformly detected in the undifferentiated cells and it decreased throughout differentiation in both systems. In the “pellet” culture, the type II collagen gene showed a very rapidly increase and decrease after 7 days in relation to the type X collagen gene; it was detected after 3 days and then it gradual increased to a uniform level. However, for the “alginate bead” culture, type II collagen mRNA was rapidly increased from 5 days to 14 days. The type X collagen gene was not detected in the undifferentiated mesenchymal stem cells, and it was rapidly up-regulated at 14 days in the “alginate bead” culture. Results from RT-PCR at the 3, 5, 7, and 14 days time points showed that collagen type I, II, X and aggrecan mRNAs were expressed in the cells and chondrogenesis should require a specific culture system such as a three-dimensional construct in the presence of the chondrogenic media.

Alcian blue staining and Safranin O staining

The morphological changes of the cells were examined by histological staining. The human mesenchymal stem cells that were differentiated by chondrogenesis showed a round morphology. The safranin O-positive stain and alcian blue-positive stain indicated that glycosaminoglycans were abundant and homogeneously distributed around the cells. The chondrogenic MSCs showed increasingly normalized staining of alcian blue and safranin O at 14 days in both the “alginate bead” and “pellet” cultures (Fig. 3).

Immunohistochemistry (IHC)

Immunohistochemical staining was performed on the alginate beads and pellet section. The results at 1, 7 and 14 days revealed that collagen type II was synthesized. Positive staining with the antibody specific for the cartilage markers (collagen type II) was observed and there was stronger staining at 14 days in the “alginate bead” culture than in the “pellet” culture. As shown Fig 3, the MSCs in the “alginate bead” culture had
more gradually increased type II collagen than did the in “pellet” culture.

DISCUSSION

Many researches were shown that the essential conditions for chondrogenesis from human mesenchymal stem cells were a 3-D culture, low serum, growth factors and so on. First, chondrocytes can be cultured in gels such as alginate and agarose, and also in suspension cultures that maintain the chondrocytic rounded shape. This appears to be critical for the maintenance of the phenotype of cells capable of forming a cartilaginous matrix. Second, Ballock et al. reported that the manipulation of the serum concentration to optimal levels of less than 0.1 or 0.01% in the chondrogenic differentiation culture system resulted in the formation of the features of developing cartilage architecture that had been exclusively observed in growth cartilage. Third, several studies have shown that several members of the transforming growth factor-β family have been shown to play a major role in cartilage development. Therefore, in this study, we used two representative 3-D culture systems ("alginate bead" culture and "pellet" culture), no serum, and TGF-β1 for chondrogenic differentiation.

In this paper, the chondrogenic differentiation of mesenchymal stem cells in two culture systems, the "pellet" culture and "alginate bead" culture, involves the deposition of an integrated extracellular matrix. The typical gene for articular cartilage, collagen type II, was more strongly expressed in the latter than in the former system and the specific gene of hypertrophic cartilage, collagen type X, was more rapidly expressed in the former than in the latter system.

Several monoclonal antibodies have been used...
as reagents in the isolation and characterization of mesenchymal stem cells. In general, antibodies such as SH-2 (CD105), 3 and 4 have been created against intact human mesenchymal stem cells.\textsuperscript{2,22-27} Human mesenchymal stem cells have been shown to positively express endoglin (CD105), hyaluronic acid receptor (CD44), and integrin β1 (CD29); however, they have not been shown to express early hematopoietic stem cell marker (CD34), monocyte or macrophage cell marker (CD14), and leukocyte cell marker (CD45). Another possible role for endoglin on mesenchymal stem cells may be for mediating TGF-β signaling during chondrogenic differentiation. All the TGF-β isoforms are capable of inducing mesenchymal stem cells along the chondrogenic pathway.

Articular cartilage consists of sparsely embedded chondrocytes in a specialized microenvironment made up of dense extracellular matrix components. Damaged cartilage has a limited spontaneous repair ability, which is compounded by a lack of precursor cells. Therefore, surgical procedures have been performed for the aim to supplying bone marrow-derived mesenchymal precursor cells to the damaged site by penetrating the underlying subchondral bone with the hope that the surrounding environment will provided the proper stimulus for differentiation of these cells.\textsuperscript{28} These procedures have usually resulted in fibrocartilage\textsuperscript{29} and these procedures have improved our limited understanding of the cartilage microenvironment, cells and factors that promoted cartilage repair.\textsuperscript{30}

The extent to which the phenotype of the mesenchymal stem cells that differentiated along a chondrogenic pathway resembled the phenotype of articular cartilage was a central question in this work. Many of the components of a cartilaginous extracellular matrix were deposited by differentiating mesenchymal stem cells that were exposed to TGF-β\textsubscript{3}, including aggregan, type II collagen, and type IX collagen. Type X collagen, which has an established association with hypertrophic cartilage, was a component of the matrix in this differentiation system.\textsuperscript{31,32} However, in the "pellet" systems, there has been an increasing amount of type X collagen observed as a component of the growth plate. Differentiating mesenchymal stem cells showed the rapid upregulation of aggregan mRNA. Aggrecan is abundantly expressed in cartilage during limb development and it is maintained at high levels through adulthood in all cartilage types.\textsuperscript{31,32} The expression of type II collagen appeared rather late in the process of matrix assembly in both systems. The expression of type II collagen during chondrogenesis may indeed indicate that they are functionally linked. That is, the "alginate bead" culture shows an earlier and more continuously expression of type II collagen than that of the "pellet" culture.

Expressions of type I collagen was noted to proceed that of type II collagen. Type II and X collagen are co-distributed in hypertrophic human cartilage with the exclusion of type I collagen. The expression of type II collagen is low in immature cartilage and it increases with maturation. For these reasons, the "alginate bead" culture system represents a useful in vitro system to study the early steps involved in the development of human cartilage. The cells also synthesized the extracellular proteoglycan matrix, as judged by alcian blue and safranin O staining, which confirms the characteristics of cartilage cells.\textsuperscript{32,33}

Finally, in the present study, the expressions of type II collagen and aggregan were increased with differentiation; those expressions in the "alginate bead" culture were similar to or higher than those expressions of the "pellet" culture, as was measured by RT-PCR and immunohistochemistry. However, the expression of type X collagen in the "alginate bead" system was lower than that in the "pellet" systems. Therefore, the "pellet" culture system might be better suited to conditions that are formatted to the early development and growth of the cartilage plate, and the "alginate bead" culture system might be the proper conditions that are similar to phenotypical articular cartilage, and so they both have a substantial clinical application after the differentiation of the chondrocytes.

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