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TGF-β1 Directs Chondrogenic Differentiation of Monocytic Precursor Cells

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Abstract

Bone marrow consists of two types of stem cell populations, the hematopoietic stem cell (HSC) and the mesenchymal stromal cell (MSC). While MSCs have been demonstrated to have the capacity to differentiate into osteoblasts, chondrocytes, and adipocytes, recent studies by our laboratory have demonstrated an HSC origin for osteoblasts, cancer-associated fibroblasts, and immature adipocytes, revealing the ability of HSCs to give rise to cell types outside of the hematopoietic lineage. Previous *in vivo* studies in our laboratory using a clonal cell transplantation model, demonstrate the ability of HSCs to give rise to hypertrophic chondrocytes during non-stabilized fracture repair. This work has led to the hypothesis that the HSC is an alternate source for chondrocytes. To begin addressing this hypothesis, *in vitro* studies were performed to determine appropriate culture conditions for HSC-derived chondroprogenitors. The importance of identifying alternate sources for chondrocytes is high, as there are currently no widely successful standard therapies for cartilage regeneration. Alcian Blue staining was used as an indicator of chondrogenic potential in determining glycosaminoglycan production. In addition to this, nodule formation was assessed in cultures in serum-containing chondrogenic media. More specific studies involved monocytic precursor cells cultured in serum-free conditions and directed towards the chondrogenic lineage via transforming growth factor beta-1 (TGF-β1). After induction, positive expression of the cartilage specific markers, Aggrecan and Collagen II, along with negative expression of F4/80, suggested chondrogenic differentiation. Future work will be aimed at delineating the mechanisms and factors governing HSC differentiation and maturation to chondrogenic lineages, with the potential to enhance stem cell therapies for cartilage repair.

Keywords Hematopoeitic stem cell; Monocyte; Chondrogenesis; Differentiation; TGF-β1

Introduction

Given that articular cartilage is avascular in nature; its regenerative capabilities are significantly hindered. Damage to articular cartilage leads to degeneration of the tissue and often to osteoarthritis. As current procedures and treatments have failed to produce consistent hyaline cartilage and restore the original architecture of articular cartilage tissue, research has examined the possibility of stem cell therapies to promote proper cartilage regeneration [1]. Bone marrow-derived stem cells, in particular, have demonstrated multilineage potential and hold great promise for tissue engineering. Although many of these studies have focused on mesenchymal stem cells (MSCs), clinical trials have resulted in limited utility of MSCs for repair of cartilage following insult. Due to this, the ability of other stem cell populations to give rise to chondrocytes warrants further study. Past research has indicated a myeloid lineage for other mesenchymal cell types, including osteoblasts/osteocytes [2,3] and adipocytes [4,5]. Studies from our laboratory based on transplantation of a clonal population of cells derived from a single sorted HSC revealed the ability of the HSC to contribute to the chondrocyte population *in vivo* in both articular cartilages during normal development and hypertrophic chondrocytes during non-stabilized fracture repair [2].Human studies have identified a peripheral blood monocyte-derived "programmable cell of monocytic origin" (PCMO) that could be induced to express collagen II, providing another example of the ability of the myeloid lineage to produce cells of the chondrogenic lineage [6]. In the present study, the *in vitro* chondrogenic potential of monocytic precursor-derived cells was examined in an effort to explore a new source of cells to be considered for cartilage regeneration. HSC-derived cells that could be easily harvested from bone marrow aspirate or from peripheral blood collections that also have the potential to be expanded and differentiated toward a chondrogenic phenotype *in vitro* could greatly impact the field of cartilage research.

Material and Methods

Isolation and culture of articular cartilage

Articular cartilage was isolated for digest or explant culture by removal of the femoral and tibial condyles of 5-9 week old C57Bl/6 CD45.1 or enhanced green fluorescent protein (EGFP) CD45.2 male or female mice. The cartilage pieces were incubated at 37°C and underwent collagenase digestion by collagenase D (Roche) at a concentration of 3 mg/mL for 30-60 minutes. The solution was collected and pipetted successively through decreasing pipette sizes in order to disperse cell aggregates. The solution was then filtered through a sterile 40 μm cell strainer and collected for centrifugation.

Cells were resuspended and plated in Dulbecco's Modified Eagle Medium (DMEM) (Gibco), 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals), 1% Penicillin/Streptomycin (Atlanta Biologicals or Quality Biologicals), supplemented with 2 mM L-glutamine (Sigma), and plated on 8-well fibronectin coated plates. For explant cultures, the femoral condyle was collected from the digest dish after incubation and plated on an 8-well fibronectin coated plate in DMEM/10% FBS/1% PS, supplemented with 2 mM L-glutamine. Explant culture was used as a means of harvesting residual articular chondrocytes that were not collected by the digestion alone. For explant culture, the femoral condyle was placed in a single well of an 8-well fibronectin plate. Media was changed every 2-3 days for all cultures and the cells were cultured for 2-3 weeks. The method of harvesting and culturing the articular cartilage was adapted from an established protocol [7].

Isolation and culture of monocytic precursor cells

Bone marrow was isolated from the femurs and tibias of 5-9 week old C57BL/6 or EGFP male or female mice by flushing medullary cavities with an insulin syringe. Mononuclear cells were collected from the bone marrow by Lympholyte-M (Cedar Lane Tech) density centrifugation, plated on T75 tissue culture flasks and incubated for 24 hrs in alpha minimum essential medium (αMEM) (Gibco), 15% FBS (Atlanta Biologicals), 1% PenStrep (Atlanta Biologicals) supplemented with 15 ng/mL IL-3 (BioAbChem) and 1 ng/mL macrophage-colony stimulating factor (M-CSF) (BioAbChem) to stimulate HSC differentiation toward myeloid progenitor cells and macrophages, respectively. After 24 hrs, the non-adherent cells were collected, centrifuged at 2300 rpm for 10 minutes, and underwent Pronase digestion with a 0.02% Pronase solution for 15 minutes at 37°C. The cell suspension was then layered on 10 mL horse serum (Atlanta Biologicals) on ice for 15 minutes. Cells were then removed, layered onto another 10 mL of horse serum, centrifuged at 2800 rpm for 10 minutes, and re-plated on a T75 flask in the αMEM media described above. After 2 days, the non-adherent cells were again collected and the above Pronase treatment was repeated. Cells were then plated in one of the following chondrogenic medias: DMEM supplemented with 1% ITS (Sigma), 1% Pencillin/Streptomycin, 2 mM L-glutamine (Sigma), 0.1 mM ascorbic acid (Wako), and 10 ng/mL transforming growth factor beta-1 (TGFβ-1) (R&D systems) or DMEM supplemented with 10% FBS, 1% PS, 2 mM L-glutamine, 0.1 mM ascorbic acid and 10 ng/mL TGF-β1 at a density of 2×10^6 or 2.5×10^6 cells/well on 8-well fibronectin plates. Cells were cultured for 6 weeks, and media was changed every 2-4 days.

Alcian blue staining

To evaluate cartilage-specific proteoglycan synthesis, sulfated glycosaminoglycans (GAGs) were visualized by staining with Alcian Blue. After 21 days of culture, cells were washed with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature, and then washed 3-5 times with distilled water. The cells were then stained with Alcian Blue (1% in 3% acetic acid, pH 2.5, 8XG solution-Sigma) for 30 minutes and rinsed with distilled water until the water ran clear (about 5 rinses). Staining was assessed visually using a Nikon Eclipse Ti Microscope.

Immunofluorescent staining

Cells were fixed in 4% paraformaldehyde in PBS for ten minutes at room temperature. Cells were washed with PBS containing 0.05% sodium azide three times for a total of 15 minutes. For aggrecan staining, cells were incubated with 5×10^{-5} U/μl Chondroitinase ABC for three hours at 37°C post-fixation then washed three times for a total of 15 minutes with PBS-Az prior to permeabilization. Cells were then permeabilized with Triton X-100 (0.02% in PBS) for ten minutes. Cells were washed with PBS-Az three times for a total of 15 minutes and then blocked in 5% donkey serum in 3% BSA-PBS for thirty minutes at room temperature. Block was removed, and the primary antibody was added (Rabbit-anti mouse Collagen II polyclonal antibody at 1:50 or Rabbit- anti-Aggrecan polyclonal antibody at 1:100, Millipore) for 45 minutes at room temperature. Cells were washed three times for a total of 15 minutes with PBS-Az and then blocked for 30 minutes in 5% donkey serum in 3% BSA-PBS. The secondary antibody, Donkey anti-Rabbit Cy3 (Jackson), was added at a 1:100 dilution for thirty minutes at room temperature. Cells were washed three times for a total of 15 minutes with PBS-Az. Hoechst (Invitrogen) was added at a 1:25000 dilution in PBS for eight minutes. Cells were washed three times for a total of 15 minutes with PBS-Az, and were then mounted and cover-slipped. Immunofluorescence was visually assessed using a Nikon 90i microscope with digital camera.

Results

HSC-derived monocytic precursor cells demonstrate glycosaminoglycan production with chondrogenic induction and TGF-β1 supplementation.

To determine if HSC-derived cells could contribute to the chondrogenic lineage *in vitro*, monocytic precursors were isolated from the bone marrow and cultured under chondrogenic conditions. Induced monocytic precursor cells were stained with Alcian Blue to demonstrate glycosaminoglycan deposition after six weeks of culture in serum-containing media. Positive staining indicated the presence of glycosaminoglycans and potential chondroblasts (Figure 1).

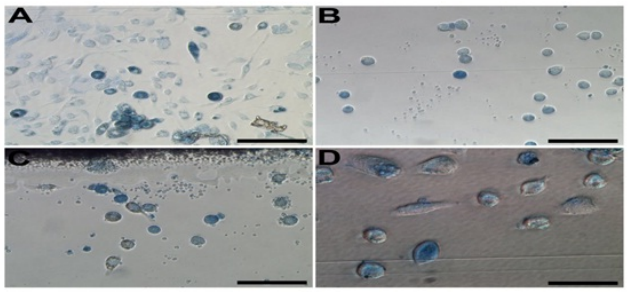

Figure 1: Monocytic precursor cells demonstrate glycosaminoglycan production with chondrogenic induction and TGF-β1 supplementation. Monocytic precursor cells cultured in chondrogenic media for six weeks and stained with Alcian Blue. Positive blue staining indicates presence of glycosaminoglycans. (A) Cells cultured in serum-containing media: DMEM/10% FBS/ 1% PS supplemented with 2 mM L-glutamine. (B-D) Cells cultured in serum free media: DMEM/ 1% ITS/ 1% PS supplemented with 2 mM L-glutamine, 0.1 mM AA, 10^{-7} dexa, and 10 ng/mL TGF-β1. Bar in (A-C)=100 μm. Bar in (D)=50 μm.

To determine if TGF-β1 played a role in promoting chondrogenic differentiation, monocytic cells were cultured in serum-free media supplemented with TGF-β1 for six weeks (Figure 1B-1D). As indicated

by positive Alcian Blue staining, TGF-β1 promoted glycosaminoglycan deposition.

In order to confirm that the positive Alcian Blue stain was indeed the result of differentiation to the chondrogenic lineage and not due to non-specific staining, monocytic precursor cells were collected after the second Pronase treatment, plated in αMEM/1% FBS at a concentration of 1×10^6 cells/well of an 8-well fibronectin plate for 2 hrs, and fixed and stained with Alcian Blue. Negative staining of these cells confirmed that the positive staining in cells resulting from culture in chondrogenic differentiation media were due to directed differentiation toward a chondrogenic lineage (Supplementary Figure 1).

As a positive control for morphology and growth patterns associated with harvested articular chondrocytes *in vitro* as well as for Alcian Blue staining, articular chondrocytes were harvested from 5-9 week old mice, plated and cultured as described above. Within the first week, the cells began to display an elongated fibroblast-like morphology. After two to three weeks in culture, both cells isolated from the digest and the explant showed formation of multiple cartilaginous nodules. Positive Alcian Blue staining indicated glycosaminoglycan deposition, a marker of chondrogenic differentiation (Supplementary Figure 2).

Monocytic precursor derived cells form cartilaginous nodules in serum-containing media supplemented with TGF-β1

Monocytic precursor-derived cells under DMEM/10% FBS/ 1% PS supplemented with 2 mM L-glutamine, 0.1 mM AA, and 10 ng/mL TGF-β1 culture conditions demonstrated cell matrix spreading by two weeks in culture and nodule formation by the end of the six week culture period (Figure 2).

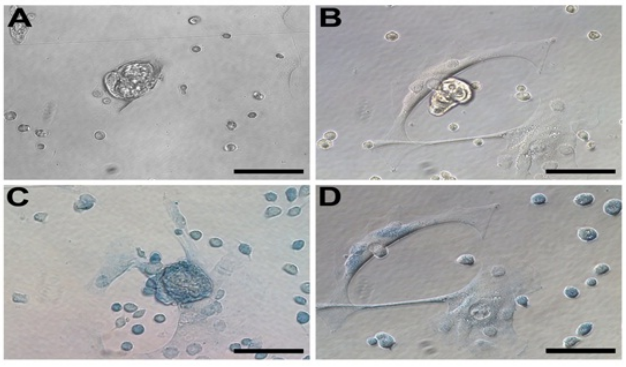

Figure 2: Monocytic precursor cells demonstrate cartilaginous nodule formation with chondrogenic induction and TGF-β1 supplementation. Monocytic precursor cells cultured for six weeks in serum-containing chondrogenic media: DMEM/ 10% FBS/ 1% PS, supplemented with 2 mM L-glutamine and 10 ng/mL TGF-β1. Negatively stained cells were also observed, confirming that the starting population of cells was not clonal. (A) Representative nodule at week four of culture–mono filter. (B) Nodule and matrix before staining–color filter. (C) Stained cartilaginous nodule. (D) Stained matrix. Bar=100 μm.

The temporal pattern in which these cells changed morphology and formed cartilaginous nodules was similar to those observed in primary articular cartilage cultures (compare Figure 2 and Figure S2). Cells cultured under these conditions stained positive for Alcian Blue, even in cultures lacking gross nodule formation.

Monocytic precursor derived cells express markers of chondrocytes after chondrogenic induction

Collagen II and aggrecan are major components of the extracellular matrix of cartilage and are produced by the resident articular chondrocytes. Production of collagen II indicates formation of hyaline cartilage, while production of a majority of collagen I indicates formation of fibrocartilage. Thus, the presence of collagen II and aggrecan in the monocytic precursor-derived cultures was examined by immunofluorescence after culture under chondrogenic conditions. Monocytic precursor-derived cells cultured under serum-free conditions and supplemented with TGF-β1 demonstrated expression of aggrecan and collagen II six weeks post-induction (Figure 3, panels B and E, respectively).

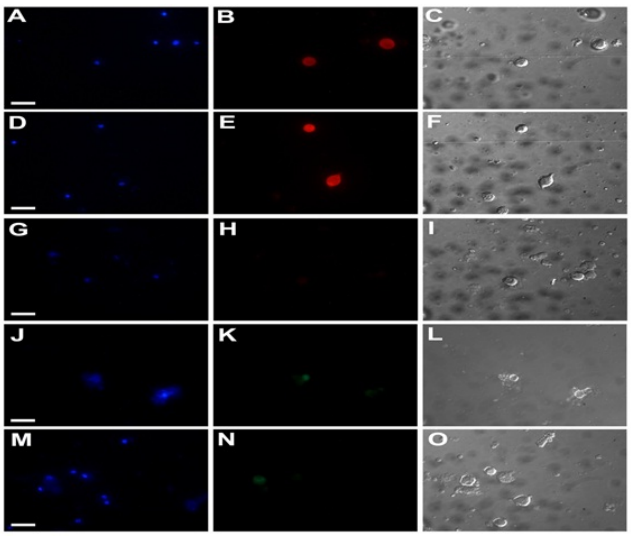

Figure 3: Monocytic precursor cells demonstrate aggrecan and collagen II production with chondrogenic induction and TGF-β1 supplementation. Immunofluorescent staining of monocytic precursor cells cultured for six weeks in serum-free media: DMEM/1% ITS/ 1% PS supplemented with 2 mM L-glutamine, 0.1 mM AA, and 10 ng/mL TGF-β1. (A,D,G,J,M) Hoechst nuclear stain. (B) Aggrecan staining. (C,F,I,L,O) DIC images. (E) Collagen II staining. (H) Secondary only control for Aggrecan and Collagen II staining. (K) F4/80 staining. (N) Secondary only control for F4/80 staining. Bar=50 μm.

Importantly, these cells lacked expression of F4/80, a mature monocyte/macrophage marker, as assessed by immunofluorescence (Figure 3K). The presence of both collagen II and aggrecan proteins within these monocytic precursor-derived cells further indicates that these cells have begun chondrocyte differentiation. In order to further confirm that the isolated myeloid precursors were actually myeloid in nature and did not express cartilage markers prior to being cultured under chondrogenic conditions, cells were collected after the second

Pronase treatment as before, plated 1 × 10^6 cells in αMEM/1%FBS for 2 hours, then fixed in 4% paraformaldehyde for ten minutes and stained for F4/80, collagen II, and aggrecan (Figure 4).

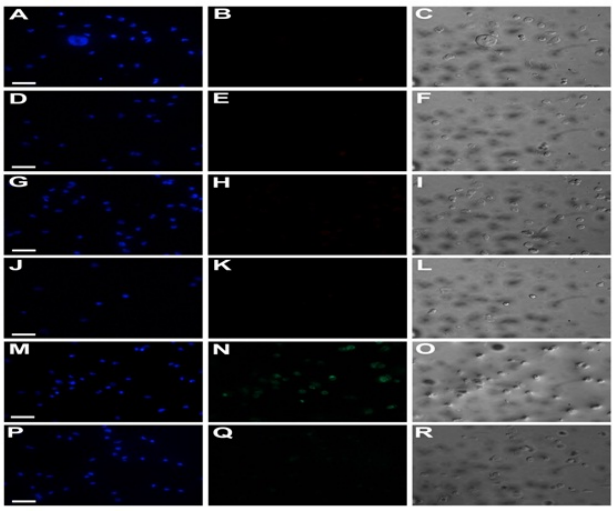

Figure 4: Monocytic precursor cells are negative for aggrecan and collagen II prior to chondrogenic induction. Monocytic precursor cells prior to chondrogenic induction. (A,D,G,J,M,P) Hoechst nuclear stain. (B) Aggrecan staining. (C,F,I,L,O,R) DIC images. (E) Secondary only control for Aggrecan staining. (H) Collagen II staining. (K) Secondary only control for Collagen II staining. (N) F4/80 staining. (Q) Secondary only control for F4/80 staining. Bar=50 μm.

Cells were positive for expression of F4/80 and negative for expression of collagen II and aggrecan, validating that these cells were from the myeloid lineage and did not non-specifically express these cartilage specific markers before induction. Together, these findings demonstrate monocytic precursor-derived cells were able to differentiate into collagen II and aggrecan-producing chondrocytes under the direction of TGF-β1. The expression of these proteins confirms that HSC-derived cells are able to differentiate into chondrogenic cells via the myeloid lineage. Lack of the F4/80 marker reflects loss of this myeloid marker during the differentiation period.

Discussion

Stem cells are an appealing avenue for use in cartilage regeneration due to their differentiation and expansion potentials [8]. Using purified HSCs as a source for chondrogenic progenitors with the capability of chondrogenic differentiation holds great clinical appeal, as they can be isolated with a high degree of homogeneity and are well defined. However, expansion of HSCs is still a major challenge in the field. Thus, the use of HSC-enriched fractions or downstream progenitors must be considered. Bone marrow is an ideal tissue that could provide an easily obtainable source of HSC-derived cells to differentiate into chonodrocytes to be utilized for therapeutic purposes [6]. This would provide an alternate source of chondrocytes other than MSCs, which have not been as efficacious in the clinical setting as initially thought. Other studies also suggest a possible hematopoietic origin for chondrocytes. Kuwana et al. has identified a monocyte precursor population termed monocyte-derived mesenchymal progenitor (MOMP) that has the ability to differentiate into chondrocyte-like cells [9]. Although an adherent cell population from the peripheral blood was utilized, these cells were shown to have a monocyte origin (CD14+) and to express CD45, CD34, and type I collagen. MOMPs showed co-expression of CD45 and the chondrogenic transcription factor Sox-9 after one week in culture with TGF-β1 and collagen II production after three weeks. In addition to this, a study conducted by Shafer et al. suggests a myelo-monocytic origin of chondrocytes [10]. After induction of bone formation, cells were positive for both smooth muscle actin (SMA) and CD68, a myeloid-specific marker; however, these cells lost expression of these markers upon the up-regulation of Sox9 and the onset of chondrogenesis. Furthermore, the β-galactosidase activity in the chondrocytes indicated myeloid origin due to the utilization of a specific Cre/lox system in which a myeloid-specific promoter is driven by Cre recombinase and irreversibly unblocks β-galactosidase in cells of myeloid origin. In this study, we have identified the ability of murine monocytic precursors to differentiate down the chondrogenic lineage, as demonstrated by expression of collagen II, aggrecan, and secretion of glycosoaminoglycans, with the potential to be cultured and expanded ex vivo to then be transplanted for repair and regeneration of cartilage defects. Given our results under serum-free conditions, we conclude that TGF-β1 alone has the power to direct differentiation of monocytic precursors toward the chondrogenic lineage and result in ex vivo nodule formation. TGF-β1 is known to up-regulate the expression of collagen II, the main component and marker of articular cartilage, which would suggest its potential in forming hyaline cartilage specifically over fibrocartilage–the ideal outcome for regenerative purposes. However, the exploration of additional growth factor contributions will aid in the discovery of the full potential of these cells concurrent with effects of TGF-β1. Thus, given the relative ease with which hematopoietic progenitor cells, including monocytic lineage cells, can be harvested from human bone marrow and the potential of autologous origin, it is likely that these cells, under the direction of TGF-β1, would be of great use in developing a cell-based intervention therapy to enhance regeneration of damaged articular cartilage. Future studies will examine the potential of monocytic progenitors, alongside administration of chondrogenic growth factors, to induce cartilage repair following injury *in vivo*.

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