LIMITATIONS OF USING AGGRECAN AND TYPE X COLLAGEN AS MARKERS OF CHONDROGENESIS IN MESENCHYMAL STEM CELL DIFFERENTIATION

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INTRODUCTION:
Tissue engineering of intervertebral discs (IVD) using mesenchymal stem cells (MSC) induced to differentiate into a disc-cell phenotype has been considered as an alternative treatment for disc degeneration. However, since it is not known how to differentiate stem cells into disc cells, one would like to differentiate stem cells into chondrocyte-like cells, embed them within a biomatrix, and test the hypothesis that these cells can produce a matrix that mimics native nucleus pulposus (NP) with properties resembling those found in healthy IVD. This is not an unreasonable supposition as cartilage and immature NP possess similar macromolecules in their matrix, but differ in their relative amounts. A high proteoglycan to collagen ratio and low aggrecan to versican ratio can be used to distinguish NP from cartilage. Pellet culture is a commonly used methodology to promote in vitro chondrogenesis of MSCs, in which the MSCs are cultured in defined chondrogenic medium [1] and supplemented with growth factors. Markers of chondrogenesis include collagen type II and aggrecan, with collagen type X being used as a marker of late stage chondrocyte hypertrophy (associated with endochondral ossification) [2]. The purpose of this study was to investigate the effects of different factors on the chondrogenic differentiation pathway using these markers to follow cell differentiation.

METHODS:
Source of stem cells – MSCs were obtained from 20-ml aspirates from the intramedullary canal of donors (60-80 years) undergoing total hip replacement for osteoarthritis using a protocol approved by the Research Ethics Committee of the Jewish General Hospital.

Preparation of human stem cells – Bone marrow aspirates were processed essentially as previously described (3). Briefly, each 20 ml of aspirate was diluted 1:1 with DMEM and layered over 1:1 ficoll (Ficoll-Paque; Pharmacia). After centrifugation at 900 x g for 30 min, the mononuclear cell layer was removed from the interface and suspended in DMEM. Cells were centrifuged at 750 x g for 15 min and resuspended in DMEM (Biomedia, Canada), 10% FBS selected for rapid growth of MSCs (Atlantic Biologicals, Norcross, GA), 100 units/ml penicillin (Biomedia), 100 µg/ml streptomycin (Biomedia), and 2 mM L-glutamine (Biomedia). The cells were counted and plated in 25 ml medium in a 176-cm² culture dish and incubated at 37°C in a 5% CO₂ humidified atmosphere. After 24 h, non-adherent cells were discarded and the adherent cells were thoroughly washed twice with DMEM.

Differentiation of stem cells – Cell differentiation was induced using a previously published method (4). Briefly, 1x10⁶ MSCs were pelleted and cultured as pellets in serum-free defined chondrogenic medium containing 1mg/ml BSA, 50 µg/ml ascorbic acid, 100 µg/ml sodium pyruvate, 1% ITS, and supplemented with 10 ng/ml TGF-β1 with or without 100 nM dexamethasone in 15-nl conical tubes. The medium was changed every 2-3 days for up to 16 days, after which they were harvested at different time points or at the endpoint for gene expression or for histological analysis.

Gene expression - Pellets were digested with Proteinase K and total RNA was extracted using Trizol (Invitrogen). Reverse transcription reaction was performed using Superscript II RNase H-RT (Invitrogen) and then amplified by PCR using gene-specific primers for types II and X collagen and aggrecan. The housekeeping gene GAPDH was used as an internal control to monitor the RNA loading. PCR products were visualized by ethidium bromide staining on 2% agarose gels.

Histology - Specimens were fixed in neutral buffered formalin and paraffin-embedded, then sections were stained with Safranine-O.

RESULTS:
Histology - After 16 days of culture in chondrogenic media supplemented with TGF-β1 the pellets stained with Safranine-O suggesting a chondrogenic phenotype rich in proteoglycan. There was no evidence of large hypertrophic chondrocytes.

Gene expression – Aggrecan message was constitutively expressed by MSCs, and levels remained relatively constant throughout the culture period (Fig. 1). Type II collagen message expression was up-regulated during the culture period. However, type X collagen message was detected early during the culture period (Fig. 2), and under some conditions type X collagen message was expressed before that for type II collagen.

DISCUSSION:
The constitutive expression of aggrecan and the early expression of type X collagen by MSCs raises the question whether they are good markers of chondrogenesis and chondrocyte hypertrophy, respectively, during MSC differentiation. While aggrecan is supposed to be a marker of chondrogenesis, type X collagen is only supposed to be expressed by chondrocytes when they become hypertrophic [2]. The expression of type X collagen before type II collagen in some cases was very surprising, since it is understood that stem cells have to be differentiated into chondrocytes before they can become hypertrophic. Thus, caution must be exercised when using aggrecan and type X collagen as markers for chondrogenesis and chondrocyte hypertrophy in association with stem cell differentiation.

REFERENCES:

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