Effect of Molecular Weight Of Hyaluronan on Chondrogenic Effect in Adipose Derived Stem Cells

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Introduction: Adipose derived stem cells (ADSCs) have attracted considerable interest for articular cartilage tissue engineering due to their self-renewing potential and chondrogenic differentiation capability. Although the chondrogenic differentiation potential of ADSCs has been reported, there is still an unmet need for the development of strategies to effectively direct ADSCs to differentiate down the chondrogenic lineage to engineer hyaline cartilage that is more similar to native cartilage. Hyaluronan is an unbranched polymer composed of repeating glucuronic acid and N-acetyl glucosamine disaccharide units. Our previous finding indicates that HA-microenvironment initiates chondrogenesis of ADSCs, and the effect is mainly through CD44 signaling. It has been reported that the interaction of CD44 and hyaluronan is strongly influenced the size of the HA ligand. The difference in HA chain length can influence both HA binding features and its functional consequences. It has been reported that HA exhibits a size-dependent stimulation of chondrogenesis of stage 24 limb mesenchymal cells. However, optimal molecular weight (M.W.) of HA for chondrogenesis of ADSCs is still undefined. This study was to search the appropriate molecular weight of HA for an optimal effect on initiating and enhancing chondrogenesis in ADSCs.

Methods: Culture of ADSCs
Human adipose derived stem cells are purchased from StemPro® Human Adipose-Derived Stem Cells (Gibco®). The Human Adipose Derived Stem Cells (ADSCs) are isolated from human lipoaspirate tissue and cryopreserved from primary cultures. Rabbit adipose derived stem cells (ADSCs) were isolated from New Zealand white rabbit. The ADSCs is cultured following a previously described method(37). The isolated ADSCs are grown at 37°C under 5% CO2 in a K-NAC medium containing Keratinocyte-SFM (Gibco BRL, Rockville, MD) supplemented with EGF-BPE (Gibco BRL, Rockville, MD), N-acetyl-L-cysteine, L-ascorbic acid 2-phosphate sequimagnesium salt (Sigma, St. Louis, MO), and 5% FBS.

Cell culture in different M.W. HA coated wells:
Purified HA(M.W. are 80kDa, 600 kDa, 2000kDa, 3000kDa) dissolved in PBS is coated on 24-well plates at concentration of 0.05mg/cm2 for 48h at 37°C. Then wells are washed twice with PBS followed by 2x10^5 cells/500µl densities of hADSCs are seeded using Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS, 1% nonessential amino acids and 100 U/ml penicillin/streptomycin (Gibco-BRL, Grand Island, NY). The medium was changed every 2 days. At every indicated time interval, cells were collected for further experimental analysis. Each experiment was performed in triplicate.

RNA isolation and real-time polymerase chain reaction (real-time PCR):
At the indicated time intervals, ADSCs will be collected. TRizol (Gibco BRL, Rockville, MD) will be used to extract the total RNA from these cells by following the manufacturer’s instructions. Briefly, 0.5-1µg of total RNA per 20 µl of reaction volume will be reverse transcribed into cDNA using the SuperScript First-Strand Synthesis System (Invitrogen). Real-time PCR reactions will be performed and monitored using
the iQTM SYBR green® supermix (Bio-Rad Laboratories Inc, Hercules, CA) and a quantitative real-time PCR detection system (Bio-Rad Laboratories Inc, Hercules, CA). The cDNA samples (2 μl, for a total volume of 25 μl per reaction) will be analyzed for the gene of interest and the reference gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). The expression level of each target gene then will be calculated as previously described (38). Four readings of each experimental sample will be performed for each gene of interest, and each experiment was repeated at least three times.

Assays for differentiation markers related to chondrogenesis:

SOX-9, Type 2 collagen, aggrecan

Sulfated glycosaminoglycan (sGAG) synthesis for cartilage specific matrix:

At every indicated time interval, ADSCs be collected and digested for 18 h at 60°C using 300μg /ml papain solution. DNA content and sulfated glycosaminoglycan (sGAG) accumulation by cells were quantified spectrofluorometrically using 33258 Hoechst dye and Sulfate Blyscan Glycosaminoglycan Assay (Biocolor, County Antrim, UK), respectively. Standard curve for DMMB assay was generated using aqueous chondroitin sulfate C (Sigma-Aldrich, St. Louis, MO) solution, with concentrations ranging from 0 to 25µg/µl.

Statistical analysis:

Each experiment will be repeated at least three times, and the data will be expressed as mean ± SE from combined data from all occasions where each experiment will be repeated. Statistical significance will be evaluated by a one-way analysis of variance (ANOVA), and multiple comparisons were performed by Scheffe’s method. A p<0.05 was considered significant.

Results: The effect of different M.W. HA on Sulfated glycosaminoglycan (sGAG) synthesis of ADSCs: To evaluate the effect of different M.W. HA on chondrogenesis of ADSCs, the sGAG synthesis was tested. Five days after ADSCs cultured on HA-coated wells, the HA-coated cultures showed more pronounced cartilage nodule formations and sGAG depositions (Figure 1). Among HA-coated groups, more pronounced cartilage nodule formations and sGAG depositions were also found in 2 million Da HA treated cultures (Figure 1). The effect of 2 million Da HA on neo-cartilage formation of ADSC in vivo: The in vivo study showed that three weeks after joint cavity implantation, the HA of 2 million M.W. possess chondrogenic induction capacity within articular cavity. The histological analysis indicated intense safranin-O staining in ADSC/HA constructs showed neo-cartilage was formed in the implanted ADSCs/HA (Figure 2).

To find the optimal M.W. HA for chondrogenesis of human ADSCs, the hADSCs were culture in different M.W. HA coated wells and the expressions of chondrogenic genes(SOX-9, Collagen type II and aggrecan) of hADSCs were detected. The result of real time PCR showed that the HA of 0.6~3 million M.W. possess the optimal chondrogenic induction capacity for hADSCs(Figure 3).

Discussion: In this study, ADSCs cultured on different M.W. HA expressed chondrogenic phenotypes including increasing sGAG synthesis. In vivo study also showed that within the joint cavity environment, the ADSCs encapsulated in HA-enriched fibrin gel were capable of promoting neo-cartilage formation in vivo. More importantly, we found that the chondrogenic effect of HA is altered by the molecular weight. 0.6~2 million M.W. of HA showed the optimal effect on promoting chondrogenesis. Based on these findings, we suggest that 0.6~2 million M.W. of HA provides better cell-niche interaction that may be an alternative choice for directing chondrogenic differentiation of ADSCs in ADSC based articular cartilage tissue engineering.
Significance: Based on these findings, we suggest that 0.6–2 million M.W. of HA provides better cell-niche interaction that may be an alternative choice for directing chondrogenic differentiation of ADSCs in ADSC based articular cartilage tissue engineering.

Figure 1. Higher M.W. of HA showed more pronounced effect on both cartilage nodule formations and sGAG depositions of ADSCs.

Figure 2. Two million M.W. of HA promotes neo-cartilage formation of ADSCs in the articular cavity.

Figure 3. HA of 0.6–3 million M.W. possess the optimal chondrogenic induction capacity for hADSCs.

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