Hyaluronan initiates chondrogenesis mainly via CD44 in human adipose-derived stem cells

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Wu S, Chen C, Chang J, Fu Y, Wang C, Eswaramoorthy R, Lin Y, Wang Y, Lin S, Wang G, Ho M. Hyaluronan initiates chondrogenesis mainly via CD44 in human adipose-derived stem cells. J Appl Physiol 114: 1610–1618, 2013. First published February 28, 2013; doi:10.1152/japplphysiol.01132.2012.—Cell-matrix adhesion is one of the important interactions that regulates stem cell survival, self-renewal, and differentiation. Our previous report (Wu SC, Chang JK, Wang CK, Wang GJ, Ho ML, Biomaterials 31: 631–640, 2010) indicated that a microenvironment enriched with hyaluronan (HA) initiated and enhanced chondrogenesis in human adipose-derived stem cells (hADSCs). We further hypothesize that HA-induced chondrogenesis in hADSCs is mainly due to the interaction of HA and CD44 (HA-CD44), a cell surface receptor of HA. The HA-CD44 interaction was tested by examining the mRNA expression of hyaluronidase-1 (Hyal-1) and chondrogenic marker genes (SOX-9, collagen type II, and aggrecan) in hADSCs cultured on HA-coated wells. Cartilaginous matrix formation, sulfated glycosaminoglycan, and collagen productions by hADSCs affected by HA-CD44 interaction were tested in a three-dimensional fibrin hydrogel. About 99.9% of hADSCs possess CD44. The mRNA expressions of Hyal-1 and chondrogenic marker genes were upregulated by HA in hADSCs on HA-coated wells. Blocking HA-CD44 interaction by anti-CD44 antibody completely inhibited Hyal-1 expression and reduced chondrogenic marker gene expression, which indicates that HA-induced chondrogenesis in hADSCs mainly acts through HA-CD44 interaction. A 2-h preincubation and coculture of cells with HA in hydrogel (HA/fibrin hydrogel) not only assisted in hADSC survival, but also enhanced expression of Hyal-1 and chondrogenic marker genes. Higher levels of sulfated glycosaminoglycan and total collagen were also found in HA/fibrin hydrogel group. Immunocytochemistry showed more collagen type II, but less collagen type X, in HA/fibrin than in fibrin hydrogels. Our results indicate that signaling triggered by HA-CD44 interaction significantly contributes to HA-induced chondrogenesis and may be applied to adipose-derived stem cell-based cartilage regeneration.

human adipose-derived stem cells; hyaluronan-CD44 interaction; articular cartilage repair; tissue engineering

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DAMAGED ARTICULAR CARTILAGE has a limited capacity for self-repair (17). Cell-based tissue engineering provides a novel strategy to treat cartilage defects (37). Due to the poor proliferation capability and de-differentiation of chondrocytes caused by in vitro expansion, mesenchymal stem cells (MSCs) have attracted interest for possible clinical use. The MSCs possess self-renewal and multilineage differentiation properties, which can be induced to chondrocytic, osteoblastic, and adipocytic lineages (6, 16, 25, 47).

Hyaluronan (HA) is one of the main components of extracellular matrix in articular cartilage (29). The surface antigen CD44 is the main receptor of HA, and the interaction between HA and CD44 (HA-CD44 interaction) on chondrocytes is crucial for the maintenance of cartilage homeostasis (1, 3, 19, 20). Our laboratory’s previous study (43) showed that an HA-enriched microenvironment initiates and enhances chondrogenesis of human adipose-derived stem cells (hADSCs). Therefore, we further hypothesize that the HA-induced chondrogenesis of hADSCs is mainly due to the HA-CD44 interaction in hADSCs.

A traditional two-dimensional (2D) monolayer culture has been employed to investigate cellular differentiation and extracellular matrix deposition in vitro (37). In tissue engineering, using a three-dimensional (3D) culture system may provide an appropriate niche, scaffolding, and environmental bioactive signals for the cells. Fibrin has been extensively used as a scaffold clinically (8). Accordingly, we first examined the effect of the HA on initiation of chondrogenesis in hADSCs by culturing the cells in 2D HA-coated wells and further tested the influence on cartilaginous matrix formation in a 3D fibrin hydrogel.

MATERIALS AND METHODS

Isolation and culture of hADSCs. After obtaining informed consent from all patients and approval from the hospital ethics committee (KMU-IRB-970267), leftover subcutaneous adipose tissue was acquired from patients undergoing orthopedic surgery. The hADSCs were isolated from human subcutaneous adipose tissue following a previously described method (7, 23, 40, 41). The cells used in this study were isolated from three different donors, including two women...
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and one man. The ages of two female donors are 34 and 65 yr old. The age of the male donor is 18 yr old. The isolated hADSCs were cultured and grown in 37°C under 5% CO₂ in a K-NAC medium containing keratinocyte-SFM (Gibco BRL, Rockville, MD) supplemented with EGF-BPE (Gibco BRL), N-acetyl-L-cysteine, l-ascorbic acid 2-phosphate sequimagnesium salt (Sigma, St. Louis, MO), and 5% FBS (23). Each experiment was performed using adipose-derived stem cells (ADSCs) from individual donor and repeated at least three times. Data merged from all the examinations were analyzed for statistical significance.

**HA receptor (CD44) detection by flow cytometry.** Flow cytometry analysis was used to examine the presence of CD44 on the cell surface of hADSCs. Following the manufacturer’s instructions, the hADSCs were harvested from the culture dishes by treating them with 0.25% trypsin/EDTA in phosphate-buffered saline (PBS). One million hADSCs were suspended in 500 µl of PBS containing 20 µg/ml of an antibody. Fluorescein isothiocyanate (FITC)-conjugated antibodies (Becton Dickinson, Franklin Lakes, NJ) targeted against CD44 were obtained from Becton Dickinson. As an isotype control, FITC-conjugated nonspecific mouse IgG (Becton Dickinson) was used. After incubation for 20 min at 4°C, the cells were washed with PBS three times and then suspended in 1 ml of PBS for analysis. Cell fluorescence was detected using a flow cytometer (FACS Vantage SE, Becton Dickinson) with a 525-nm filter for green FITC fluorescence, and the data were analyzed using WINMDI software.

**Cell culture on HA-coated wells.** To prepare HA-coated wells, purified HA (grade FCH-200, molecular mass = 2–2.1 Mda) (Kibun Food Chemicals, Tokyo, Japan) dissolved in PBS was coated on 24-well plates (0.5 mg/cm²) for 48 h at 37°C, followed by two washes with PBS (43). The HA/ADSCs were seeded at a density of 1 × 10⁵ cells per 500 µl of a basal medium (no chondro-induction growth factor added) made from Dulbecco’s modified Eagle’s medium containing 10% FBS, 1% nonessential amino acids, and 100 U/ml of penicillin and streptomycin (Gibco-BRL, Grand Island, NY). The culture medium in the plates was changed every 2 days. At every indicated time interval, cells were collected for further experimental analysis.

**Receptor binding inhibition assay.** To investigate the correlation between HA-CD44 interaction and the initiation of chondrogenesis in hADSCs in an HA-enriched microenvironment, the HA receptor binding inhibition assay was performed by treating hADSCs with CD44-blocking antibody. Anti-CD44 antibody (Clone 5F12, Thermo Scientific) was used to block the binding of HA to the CD44 receptor (22). The hADSCs were pretreated with 20 µg/ml of anti-CD44 antibody for 2 h at 37°C under 5% CO₂ (39). After 2 h of incubation, the pretreated cells were then resuspended in 1 ml of the basal medium (no chondro-induction growth factor added) plus 20 µg/ml of anti-CD44 antibody and were seeded in HA-coated wells. The medium was changed every 2 days. At each indicated time interval, cells were collected for further experimental analysis.

**Isolation of fibrin from rabbits.** Isolation of fibrin from a rabbit was performed following a previously described method (33). The animal study was approved by the Animal Experiment Committee of Kaohsiung Medical University, Taiwan. Male New Zealand White rabbits weighing 2.5–3 kg were used. The rabbits were first anesthetized with xylazine (5 mg/kg) and ketamine (45 mg/kg) (34). The anesthesia was supplemented with a subcutaneous injection of 0.5% lidocaine hydrochloride. After anesthesia, fresh rabbit blood was collected from the carotid vessel, and the collected blood was immediately mixed with 10% (wt/vol) sodium citrate at a ratio of 9:1. The citrated blood was stored on ice, and then plasma was separated by centrifugation at 4°C at 600 g for 20 min. A cryo-precipitation method was used for fibrin preparation (33). Briefly, the plasma was frozen for 2 h at −20°C and then thawed at 4°C, and the freeze/thaw cycle was repeated three times. Precipitated fibrin was separated from plasma by centrifugation at 1,600 × g for 5 min. The separated fibrin was dissolved in PBS (100 mg/ml) and stored at −80°C until needed.

**hADSCs and HA encapsulation in 3D fibrin hydrogel carrier.** The hADSCs were preincubated in 1% HA solution (5 × 10⁵ cells/30 µl) or in PBS (control) for 2 h at 37°C under 5% CO₂. After preincubation, every 30 µl of hADSCs (5 × 10⁵ cells) suspended in HA or PBS were mixed with 120 µl of fibrin solution (100 mg/ml) and then placed in a Teflon mold 5.5 mm in depth and 5.5 mm in diameter. Then 40 µl of bovine thrombin (300 U/ml) in 40 mM CaCl₂ was added to the mold and were mixed well with the cell/fibrin solution. This mixture was incubated at room temperature for 15 min to form a hydrogel. After gelation, the 3D fibrin hydrogel carriers containing hADSCs with HA (HA/fibrin hydrogel) or hADSCs with PBS (fibrin hydrogel) were removed from the Teflon molds, transferred to a 24-well plate, and cultured with 1 ml of chondrogenic medium [containing 10 ng/ml transforming growth factor-β] (R&D Systems, Minneapolis, MN), 50 µM l-ascorbate-2-phosphate, and 6.25 µg/ml insulin] (23, 43). The medium was changed every 2 days. At every indicated time interval, the constructs were collected for further experimental analysis.

**Cell survival in 3D HA/fibrin hydrogel carrier.** Live/dead images of HA/fibrin and fibrin hydrogel constructs were taken 4 h after cells were encapsulated. The medium was discarded, and the constructs were washed twice with PBS. Cell survival was evaluated based on the integrity of the cellular membrane using a LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR), which contains calcein-AM (live dye, green) and ethidium homodimer-1 (dead dye, red). A dye solution was made with 0.5 µl of calcein-AM and 2 µl of ethidium homodimer-1 in 1 ml of the standard medium. A slice of the construct was incubated in 1 ml of the LIVE/DEAD dye solution in a 3.5-mm dish for 30 min. Fluorescence microscopy was performed using a fluorescein optical filter to detect calcein-AM and a rhodamine optical filter to detect ethidium homodimer-1.

**RNA isolation and real-time PCR.** At the indicated time intervals, hADSCs were collected from wells or 3D fibrin hydrogel carriers. TRIZol (Gibco BRL, Rockville, MD) was used to extract the total RNA from these cells by following the manufacturer’s instructions. Briefly, 0.5–1 µg of total RNA per 20 µl of reaction volume were reverse transcribed into cDNA using the Superscript First-Strand Synthesis System (Invitrogen). Real-time PCR reactions were performed and monitored using the IQ™ SYBR green supermix (Bio-Rad Laboratories, Hercules, CA) and a quantitative real-time PCR detection system (Bio-Rad Laboratories). The cDNA samples (2 µl, for a total volume of 25 µl per reaction) were analyzed for the gene of interest, and the reference gene glyceraldehyde-3-phosphate dehydrogenase. The expression level of each target gene was then calculated as previously described (24). Four readings of each experimental sample were performed for each gene of interest, and each experiment was repeated at least three times. The primer sequences used are shown in Table 1.

**Sulfated glycosaminoglycan synthesis.** At indicated time intervals, HA/fibrin and fibrin hydrogels were collected and digested for 18 h at 60°C using 1 ml of 300 µg/ml papain solution. DNA content and sulfated glycosaminoglycan (sGAG) accumulations were quantified with a spectrophotometer using 33258 Hoechst dye and dimethylmethylen blue, respectively (13, 42). Standard curves for the dimethylmethylen blue assay were generated using an aqueous solution of chondroitin sulfate C (Sigma-Aldrich, St. Louis, MO) with concentrations ranging from 0 to 25 µg/ml. 

**Total collagen synthesis.** To measure collagen synthesis, Sirius Red dye (Direct Red, Sigma) was used to stain total collagen (38). At the indicated time intervals, the HA/fibrin and fibrin hydrogels were collected and lyzed using the freeze-thaw method (28). The cell extracts (50 µl/well) were placed in 96-well plates and kept in a dry incubator at 37°C for desiccation. Each well was washed with 200 µl of distilled H₂O three times for 1 min/wash. Then 100 µl of 0.1% Sirius Red stain (0.05 g Sirius Red powder per 50 ml picric acid) were added to each well, and the wells were kept at room temperature for 1 h. The unattached stain was removed, and the plate was washed five
times with 200 μl of 0.1 M HCl. The attached stain was extracted by mixing well with 200 μl of 0.1 M NaOH for 5 min. The attached extracted stain was placed into a second plate to read the absorbance at 540 nm using a microplate reader.

**Immunohistochemistry.** The effect of HA-CD44 on the formation of a cartilaginous matrix by hADSCs cultured in HA/matrigel and fibrin hydrogels was evaluated after 21 days in chondrogenic medium. To assess the presence of cartilage-specific matrix proteins, the HA/fibrin and fibrin hydrogels were fixed overnight using 4% paraformaldehyde in PBS (pH 7.4) at 4°C and transferred to 70% ethanol until the next processing step. The hydrogels were embedded in paraffin and were sliced into 2-μm-thick sections. To detect collagen type II, sections were labeled with specific primary antibodies for collagen type II (diluted 1:100, Chemicon), followed by rhodamine anti-mouse secondary antibodies (diluted 1:200, Invitrogen). The sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (diluted 1:500, Sigma) to identify cellular nuclei, which can be counted to reflect total cell number.

**Statistical analysis.** Four cultures were tested for each individual experiment. Each experiment was repeated at least three times, and the data were expressed as means ± SE from combined data from all occasions where each experiment was repeated. Statistical significance was evaluated by a one-way ANOVA, and multiple comparisons were performed by Scheffé’s method. A $P < 0.05$ was considered significant.

**RESULTS**

**Flow cytometry analysis of CD44 in hADSCs.** The results from flow cytometry analysis showed that 99.9% of the hADSC population possess CD44 (Fig. 1).

**HA-CD44 interaction of hADSCs in HA-coated cultures.** The interaction between HA-CD44 for hADSCs in an HA-enriched microenvironment was tested by examining the mRNA expression of hyaluronidase-1 (Hyal-1) in HA-coated wells (0.5 mg/cm² per well). The results showed that the mRNA expression of Hyal-1 was significantly upregulated in HA-coated cultures (Fig. 2). Compared with the control group, which did not use HA-coated wells, Hyal-1 mRNA expression was significantly increased in HA-coated cultures from day 1 to day 5 [day 1 ($P = 0.014$), day 3 ($P = 0.035$), and day 5 ($P = 0.041$)]. This result indicates that HA-CD44 interaction occurs in hADSCs cultured in an HA-enriched microenvironment (Fig. 2A).

**Initiation of chondrogenesis of hADSCs in HA-coated well.** To test the initiation of chondrogenesis by hADSCs in an HA-enriched microenvironment, the mRNA expression of chondrogenic marker genes was tested in HA-coated cultures (0.5 mg/cm²). The mRNA expression of chondrogenic marker genes (SOX-9, collagen type II, and aggrecan) was significantly upregulated in cultures on HA-coated wells (Fig. 2). Compared with the control group, upregulation of chondrogenic marker gene expression in HA-coated group was increased from day 1 to day 5 [SOX-9: day 1 ($P = 0.018$), day 3 ($P = 0.041$), and day 5 ($P < 0.0001$); collagen type II: day 1 ($P = 0.034$), day 3 ($P = 0.021$), and day 5 ($P = 0.005$); and aggrecan: day 1 ($P = 0.003$), day 3 ($P = 0.001$), and day 5 ($P = 0.002$)] (Fig. 2, B, C, and D). These results indicate that chondrogenesis in hADSCs was initiated in an HA-enriched microenvironment.

**Blockade of HA-CD44 signaling inhibits Hyal-1 expression and chondrogenic gene expression.** The Hyal-1 mRNA expression for hADSCs in HA-coated cultures was significantly

### Table 1. Primer sequences and cycling conditions for real-time PCR

<table>
<thead>
<tr>
<th>Human gene</th>
<th>PCR Primers Sequence (Forward and Reverse)</th>
<th>Annealing Temperature, °C</th>
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<tbody>
<tr>
<td>SOX-9</td>
<td>Forward: 5’-CCT CGG CGA GGT GGA CAT-3’</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-GTT GGG CAG GTA CTG-3’</td>
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</tr>
<tr>
<td>Type II collagen</td>
<td>Forward: 5’-CAA CAC TGC CAA GGT CAT-3’</td>
<td>61</td>
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<tr>
<td></td>
<td>Reverse: 5’-TCT TGG AGT GGT TGA TGT TCT-3’</td>
<td></td>
</tr>
<tr>
<td>Aggrecan</td>
<td>Forward: 5’-ACA GCT GGG GAC ATT AGT GG-3’</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-CTT TCG ACT ACA AGG AGG-3’</td>
<td></td>
</tr>
<tr>
<td>Hyaluronidase-1 (Hyal-1)</td>
<td>Forward: 5’-AAA ATA CAA GAA AGG AAT CAT GTG-3’</td>
<td>55</td>
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<tr>
<td></td>
<td>Reverse: 5’-GCG AGC ACA AGG CCT GAC-3’</td>
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<tr>
<td>GAPDH</td>
<td>Forward: 5’-TCC CTT CTC ACT ACA GCG AC-3’</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-CCC TGT TGC ATG AGC CAA ATT C-3’</td>
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<tr>
<td>Cycling conditions</td>
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<tr>
<td>Denature: 95°C for 30 s, 95°C for 4 min, followed by 35 cycles of 95°C for 10 s, 58.4–61.5°C (shown in column of Annealing Temperature) for 15 s and 72°C for 15 s</td>
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**CD44 positive**

**Isotype control**

---

**Fig. 1.** Flow cytometry analysis to confirm the presence of CD44 on the cell surface of human adipose-derived stem cells (hADSCs). The analysis showed that 99.9% of the hADSC population can be stained with CD44 positive (gray) compared with the isotype control (white) stained hADSCs.
increased, but not different from that in HA-CD44 blockade group compared with the control group (Fig. 3A). Most importantly, the results showed that the upregulated Hyal-1 mRNA expression of hADSCs in HA-coated cultures from day 1 to day 5 was completely inhibited when treated with CD44 receptors blocking antibody (HA vs. HA+CD44 blockers: day 1, P = 0.043; day 3, P = 0.015; day 5, P = 0.008) (Fig. 3A). The upregulation of chondrogenic marker genes (SOX-9, collagen type II, and aggrecan) for hADSCs in HA-coated cultures was also suppressed when HA-CD44 was blocked (SOX-9: day 3, P = 0.03 and day 5, P = 0.039; collagen type II: day 3, P = 0.043 and day 5, P = 0.03, aggrecan: day 3, P = 0.038 and day 5, P = 0.04) (Fig. 3, B, C, and D). The decrease of mRNA expression levels for chondrogenic genes caused by the CD44 blockade was as follows: SOX-9: day 3, 37% decrease, day 5, 53% decrease; collagen type II: day 3, 38% decrease, day 5, 37.5% decrease; and
aggrecan: day 3, 42.5% decrease, day 5, 54% decrease. These results indicate that HA-CD44 contributes to the initiation and enhancement of chondrogenesis for hADSCs in an HA-enriched microenvironment.

Cell survival in 3D HA/fibrin hydrogel. After a 2-h preincubation, the survival rate of hADSCs encapsulated in 3D HA/fibrin and fibrin hydrogels was examined (Fig. 4). Four hours after encapsulation, most of the ADSCs were alive (Fig. 4, B and C), and no obvious dead cells were found in both the HA/fibrin and fibrin hydrogels (Fig. 4, B–E). The amount of aggregates was not increased, but the aggregates were larger, more pronounced in the HA/fibrin hydrogels compared with the fibrin hydrogels, indicating more hADSC and extracellular matrix interactions (Fig. 4, B and C).

The HA-CD44 interaction of hADSCs cultured in 3D HA/fibrin hydrogel. The hADSCs cultured in HA/fibrin hydrogels showed higher Hyal-1 and chondrogenic gene (SOX-9, collagen type II, and aggrecan) expression than those in fibrin hydrogels after 1, 3, and 5 days of culturing in the chondrogenic medium (Fig. 5A). Chondrogenic marker gene expression was upregulated in hADSCs cultured in HA/fibrin hydrogels from day 1 to day 5 compared with fibrin hydrogels [SOX-9: day 1 (P = 0.023), day 3 (P = 0.019), and day 5 (P = 0.0284); collagen type II: day 1 (P = 0.0195), day 3 (P = 0.0239), and day 5 (P = 0.0408); aggrecan: day 1 (P = 0.0157), day 3 (P = 0.0131), and day 5 (P = 0.0476)] (Fig. 5, B, C, and D). These results confirm that HA-CD44 contributes to promoting chondrogenesis for hADSCs in HA/fibrin hydrogels (Fig. 5, B, C, and D).

sGAG and total intracellular collagen synthesis of hADSCs cultured in a 3D HA/fibrin hydrogel. The sGAG and total intracellular collagen synthesis by hADSCs in HA/fibrin and fibrin hydrogels was quantified to support the gene expression results. The result showed that hADSCs cultured in HA/fibrin hydrogels produced more sGAG than that in fibrin hydrogels.
In the present study, we further hypothesized that the HA-CD44 interaction contributed to HA-initiated chondrogenesis and the subsequent formation of a cartilaginous matrix. We found that CD44 signaling was promoted in hADSCs cultured in a HA-coated 3D environment compared with fibrin hydrogels following HA preincubation. The chondrogenesis of hADSCs was initiated, and subsequent cartilaginous matrix formation was enhanced in either HA-enriched environment or preincubation of cell with HA, indicating this effect may be due to HA-CD44 interaction. Our results further demonstrated that a blockade of HA-CD44 interaction decreased the HA-upregulated mRNA expression levels of chondrogenic marker genes, including SOX-9, collagen type II, and aggrecan. This result indicates that the HA-CD44 interaction significantly contributes to both chondrogenesis of hADSCs and subsequent cartilaginous matrix formation.

**Discussion**

Cell-matrix adhesion is one of the niche interactions that direct stem cell differentiation (11). Our laboratory's previous report indicated that an HA-enriched microenvironment initiates and enhances chondrogenesis in hADSCs (43). In the present study, we further hypothesized that the HA-CD44 interaction contributes to HA-initiated chondrogenesis and the subsequent formation of a cartilaginous matrix. We found that CD44 signaling was promoted in hADSCs cultured in both HA-coated wells and HA/fibrin hydrogels following HA preincubation. The chondrogenesis of hADSCs was initiated, and subsequent cartilaginous matrix formation was enhanced in either HA-enriched environment or preincubation of cell with HA, indicating this effect may be due to HA-CD44 interaction. Our results further demonstrated that a blockade of HA-CD44 interaction decreased the HA-upregulated mRNA expression levels of chondrogenic marker genes, including SOX-9, collagen type II, and aggrecan. This result indicates that the HA-CD44 interaction significantly contributes to both chondrogenesis of hADSCs and subsequent cartilaginous matrix formation.

Fig. 5. Real-time polymerase chain reaction analysis illustrating the interaction between the HA-CD44 interaction and initiation of chondrogenesis in hADSCs cultured in HA/fibrin hydrogel constructs: Hyal-1 (A), SOX-9 (B), collagen type II (C), and aggrecan (D). We normalized the values against expression in controls without HA at day 1, which was stated at 1. Values are means ± SE (n = 4). *P < 0.05, **P < 0.01 compared with fibrin hydrogel at each time point.

Fig. 6. Synthesis of sulfated glycosaminoglycan (sGAG; A) and total intracellular collagen (B) from hADSCs cultured within HA/fibrin and fibrin hydrogel constructs after 21 days. Values are means ± SE (n = 4). *P < 0.05, **P < 0.01 compared with fibrin hydrogel at each time point.
Increasing chondrogenic activity in MSCs is important for stem cell-based tissue engineering to repair articular cartilage. CD44 has been reported to be the main surface receptor of HA and is important for cartilage matrix assembly and retention (3, 10, 19). Our results showed that 99.9% of the hADSCs possesses CD44. This finding increases the possibility that extracellular HA may act through its interaction with CD44 in hADSCs. Upregulation of Hyal-1 has been reported to reflect the activation of CD44 signaling, which is triggered by HA binding in MSCs (26). In the 2D HA-coated culture experiment, cells cultured in basal medium expressed high gene levels of Hya-1, SOX-9, collagen type II, and aggrecan on the first day, caused by HA-CD44 interaction, while blocking the HA-CD44 interaction by antibody. The gene expressions were partially downregulated during days 1–5. Therefore, we conclude that the HA-CD44 interaction contributes to inducing and enhancing the chondrogenic differentiation of ADSCs. In the 3D fibrin hydrogel experiment, for observing
the cartilaginous matrix formation, ADSCs were cultured in the chondrogenic medium. The cultures with HA-CD44 interaction showed higher expression levels of these genes than cultures without HA-CD44 interaction. Based on these results, we conclude that HA-initiated chondrogenesis in hADSCs acts mainly via CD44-mediated signaling. Nevertheless, there may be pathways other than CD44 mediation involved in HA-initiated chondrogenesis. It has been reported that receptors like RHAMM (receptor for HA-mediated motility) and layilin also can interact with HA; however, the interactions between these receptors and HA are still not well defined (5, 9, 14). Accordingly, in this study, we focused on the research of the interaction of HA with CD44. Previous study found chondrogenesis in HA scaffolds was efficiently induced in ADSCs (18). On the other hand, previous study indicated upregulation of bone morphogenetic protein (BMP)-2 stimulates chondrogenic phenotype of intervertebral cells, including upregulation of collagen type II and aggrecan mRNA expressions and sGAG depositions. Besides, better proliferation and chondrogenic differentiation of ADSCs were obtained in the 3D HA scaffold culture compared with the micromass culture. The level of chondrogenic differentiation of ADSCs in the HA scaffold was further increased by BMP-2. The results suggested that the HA scaffold was a promising chondrogenic cell culture system of ADSCs, and that BMP-2 could potentially serve as a chondrogenic supplement for ADSCs (44). In our unpublished data, HA-CD44 interaction upregulated BMP-2 expression in hADSCs. These previous results implied that the bands may have cross talks among the intracellular signal transduction pathways for CD44-mediated and chondrogenesis-related signals, such as BMP-2 and/or transforming growth factor-β. In this stage, we only can conclude the contribution of HA-CD44 interaction to the chondrogenesis of ADSCs. The detail molecular mechanisms need to be further investigated in future.

Tissue engineering aims to create functional tissue using differentiated or stem cells and scaffolds to facilitate cell growth, organization, and differentiation (37). The traditional 2D monolayer culture may limit the cells’ ability to synthesize cartilaginous tissue because of the improper mechanical and biochemical culture conditions (36, 46). In this study, we demonstrated that the HA-CD44 interaction significantly contributes to chondrogenesis in hADSCs cultured in a 2D monolayer without any chondrogenic induction supplement. We further confirm cartilaginous matrix formation by culturing hADSCs in an HA/fibrin hydrogel following a 2-h preincubation of cells with HA. More pronounced cell aggregates were found for hADSCs that were preincubated with HA and cultured in HA/fibrin hydrogel rather than hADSCs in fibrin hydrogel. A simultaneous increase in expressions of both Hyal-1 and chondrogenic marker genes also occurred in hADSCs cultured in the HA/fibrin hydrogel, suggesting that HA-CD44 interaction-induced onset of chondrogenesis also occurred in a 3D culture system. Fibrin hydrogel was used as a scaffold in this study because it has high water content, which is similar to cartilage. Additionally, fibrin is a FDA-approved safe biomaterial for clinical applications (2, 12, 15, 21, 30–32, 35, 36, 42). We found that fibrin hydrogel can be used to encapsulate hADSCs without affecting cell survival. Our results from the 3D cultures suggest that the HA-CD44 interaction may direct stem cell differentiation to chondrocytes in an HA/fibrin hydrogel. The HA/fibrin hydrogel may be used as a scaffold for ADSC-based cartilage regeneration.

There are two major limitations in this study. First, it is not able to precisely quantify the cartilaginous matrix proteins of collagen types II and X in immunohistological study. Using Western blot analysis for the extracted proteins from fibrin/ADSCs or HA/fibrin/ADSCs construct showed lots of background noise. Instead of testing collagen II and X individually, we measured total collagen by Sirius red staining method, and further used immunohistochemical to confirm that the total collagen change is due to collagen II but not collagen X. Second, presenting cell morphology by micrographs may benefit to confirm the finding, but it is hard to have clear pictures from hydrogel 3D cultures.

In this study, we found cells preincubated with HA and cultured in an HA-enriched 3D environment significantly enhanced both total collagen and sGAG synthesis. This suggests that the enhancement of cartilaginous matrix formation in an HA/fibrin hydrogel may be mainly due to the HA-CD44 interaction. In addition, collagen type X has been reported to be the marker of hypertrophic chondrocytes, representing chondrocyte degeneration, and it may lead to calcification of engineered cartilage (4, 27). Our immunocytochemical staining results showed that cultivation of hADSCs in an HA/fibrin hydrogel increases collagen type II, but collagen type X deposition appeared to be less compared with that in a fibrin gel without HA. Based on these results, we suggest that HA, acting through the HA-CD44 interaction, may be used as a biomaterial base to promote chondrogenesis of hADSCs for better cartilage formation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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