Expression of CD44 standard form and variant isoforms in human bone marrow stromal cells

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ABSTRACT

Human Bone Marrow Stromal Cells (hBMSCs) can migrate from bone marrow to injured tissues, where they may differentiate into different types of new cells for replacement of dysfunctional cells. CD44 plays an important role in stem cell movement. The expression distribution of CD44 standard form (CD44S) and CD44 variants (CD44V) is closely related to cell movement and tissue migration. The aim of this study was to evaluate the expressions of CD44S and CD44V in hBMSCs. The hBMSCs from four human subjects were cultured in vitro. Phenotypic properties were analyzed by flow cytometry, and adipocyte and osteoblast differentiations were evaluated at passage 4. The expressions of CD44S and CD44V were examined using quantitative real-time polymerase chain reaction (q-PCR). Results showed that hBMSCs were successfully cultured, with positive expressions of markers of mesenchymal cells (CD90, CD73, CD105), and negative expressions of markers of hematopoietic cells (CD34, CD45). The cultured hBMSCs can be induced to differentiate into adipocytes and osteoblasts. Q-PCR results showed that the expression of CD44S was significantly higher than the expressions of different CD44V isoforms in different samples. These results revealed significant differences in the distributions of CD44S and CD44V gene expressions, demonstrating a dominant CD44S expression in hBMCSs.

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1. Introduction

Mesenchymal stem cells (MSCs) are multipotent stem cells with the ability to differentiate into a variety of cell types, including osteoblasts, adipocytes and chondrocytes (Abdallah and Kassem, 2008; Pittenger et al., 1999). They are capable of tissue regeneration when being introduced into different tissues in some clinical trials. MSCs may move to injury sites, a process that has been called stem cell homing. Intravenous transplantations of mesenchymal stem cells have been studied in many clinical trials for systemic diseases (Li et al., 2015; Takayanagi et al., 2015). Some receptors expressed in MSCs that were shown to be relevant for homing have been relatively well investigated and characterized (Henschler et al., 2008).

CD44, a cell surface hyaluronate receptor 1 and a cell adhesion molecule that influences cell motility, survival and proliferation, plays a key role for migration of the MSCs into damaged tissues. Sackstein et al. have revealed that CD44 renders MSCs capable of binding to E-selectin and programs human multipotent mesenchymal stromal cell trafficking into bone tissue (Sackstein et al., 2008). Zhu et al. suggested that CD44 has an important role in mesenchymal stem cell migration in the extracellular matrix (Zhu et al., 2006). Hererra et al. have revealed that the CD44 molecule was clearly required for migration of exogenous MSCs into damaged kidney tissue in a murine model (Herrera et al., 2007).

The CD44 gene can generate many functionally distinct isoforms such as the CD44 standard isoform (CD44S) and CD44 variant isoforms (CD44V) by complex alternative splicing (Xu et al.,

2015). CD44S is expressed in most cells (Misra et al., 2011), while CD44V is expressed primarily in cells during inflammation and in tumor cells (Ponta et al., 2003; Turley et al., 2002). High expressions of certain CD44 variant isoforms were reported to be involved in tumor cell metastasis (Henke et al., 1996). The expression and biological function of CD44 variant isoforms in MSCs are poorly understood. Given the related studies in the literature, we suspect that CD44 variants may play an important role in the migration of MSCs. The aim of our study was to examine the expressions of CD44S and CD44V and to better characterize the expression patterns of CD44 in hBMSCs.

2. Materials and methods

2.1. hBMSCs culture

Specimens of the bone marrow of four human patients (male, age 35–56 years old) with femoral neck fracture were procured at the *Orthopedic Medical Center of the Second Hospital of Jilin University.* hBMSCs were isolated from the bone marrow specimens, yielding the first passages. The hBMSCs of the four patients were recovered and cultured in Dulbecco's modification of Eagle's medium (DMEM) (Themo scientific, waltham, MA, USA) with low glucose and 10% fetal bovine serum (FBS, Themo scientific, Waltham, MA, USA), and were incubated at 37 °C in a 5% CO_2 humidified incubator. The hBMSCs were passaged with 0.25% trypsin-EDTA (Themo scientific, Waltham, MA, USA) when the cultured cells reached 80–90% confluence. In order to achieve the desired cell numbers, the hBMSCs have been expanded 3 times.according above culture method.

2.2. Flow cytometric analyses of hBMSCs

The phenotypic properties of hBMSCs at passage 4 were determined for the expressions of the markers CD90, CD73, CD105, CD45 and CD34 by flow cytometry. Briefly, hBMSCs (1×10^6 cells) were dissociated with 0.25% trypsin-EDTA and were washed twice with PBS buffer, then incubated with anti-human CD73/phycoerythrin (PE), anti-human CD45/fluoresceinisothiocyanate (FITC), anti=human CD34/FITC, anti-human CD105/FITC, anti-human CD90/FITC and isotype control at 4 °C for 30 min in the dark. These monoclonal antibodies were obtained from Miltenyi Biotec (Cologne, Germany). Samples were detected by the EPICSRXL (Beckman Coulter, Kraemer Boulevard Brea, CA, USA) and data were analyzed by the EXPO32 software (Beckman Coulter).

2.3. Induction of adipocyte and osteoblast differentiation

The ability of hBMSCs at passage 4 to differentiate into adipocytes and osteoblasts were examined. To induce adipogenic differ-entiation, hBMSCs are plated in 24-well tissue culture plates with 1 ml medium per well. When the cells reached 100% confluence, they were carefully aspirated off the medium from the well, and 1 ml of adipogenic induction medium (Cyagen Biosciences, Guang Zhou, CHINA) was added. After 3 days, it was replaced with adipogenic maintenance medium (Cyagen Biosciences, Guang Zhou, CHINA), and was repeated. After 3 complete cycles of induction/-maintenance, the adipocyte-induced cells were identified by staining with oil red O (Cyagen Biosciences, Guang Zhou, CHINA). To induce osteogenic differentiation, hBMSCs were plated in 24-well tissue culture plates, and when the cells reached 100% confluence, they were cultured with a osteogenic induction medium (Cyagen Biosciences, Guang Zhou, CHINA) for 21 days.The osteoblast induced cells were identified by staining with alizarin red (Cyagen Biosciences, Guang Zhou, CHINA).

2.4. Quantitative real-time polymerase chain reaction (q-PCR)

Total RNA was extracted from 1×10^6 hBMCS cells using the RNAiso Plus (Takara, Da Lian, CHINA). mRNA was reverse transcripted into cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Da Lian, CHINA). q-PCR was conducted in a LightCycler 480 real-time PCR system (Roche, Basel, Switzerland) using SYBR *Premix Ex Taq* II (Takara, Da Lian, CHINA). Beta-actin was used as a reference mRNA control. q-PCR cycling conditions were: 95 °C for 10 min, (95 °C for 15 s, 60 °C for 30 s, 40 cycles), 95 °C for 60 s, followed by a dissociation curve analysis. The primer sequences from reference Li et al. (2014) used for amplification of CD44S and CD44V and beta-actin are shown in Table 1. The IDEAS2.0 software was used to analyze the curve of PCR amplification and to calculate the C_P (Crossing point), referring to the number of cycles at which the fluorescence exceeded the threshold. The smaller the value of C_P, the higher the content of the original mRNA in the sample.

2.5. Statistical analysis

A one-way ANOVA is used to test the difference between CD44S and CD44v expression in hBMSCs.

SPSS10.0 software(SPSS Inc.) was used for all the statistical analyses. All the tests were performed at a significance level of 0.05.

3. Results and discussion

3.1. Characterization of hBMSCs

Morphologies of hBMSCs at passage 4 showed a large number of adherent and flattened fibroblast-like cells and a few small, round-shaped floating light cells in the culture (Fig. 1A). The examination

Table 1
Sequences of the CD44V and CD44S and beta-actin PCR primers.

|	Forward primer	Reverse primer	Size(bp)
CD44S	GGAGCAGCACTTCAGGAGGTTAC	GGAATGTGTCTTGGTCTCTGGTAGC	129
CD44v2	ATCACCGACAGCACAGACAGAAT	AACCATGAAAACCAATCCCAGG	110
CD44v3	TACGTCTTCAAATACCATCTCAGCA	AATCTTCATCATCATCAATGCCTG	110
CD44v4	AACCACACCACGGGCTTTTG	TCCTTGTGGTTGTCTGAAGTAGCA	104
CD44v5	TGCTTATGAAGGAAACTGGAAC	TGTGCTTGTAGAATGTGGGGT	94
CD44v6	CCAGGCAACTCCTAGTAGTACAACG	CGAATGGGAGTCTTCTTTGGGT	112
CD44v7	GCCTCAGCTCATACCAGCCATC	TCCTTCTTCCTGCTTGATGACCT	127
CD44v8	TGGACTCCAGTCATAGTATAACGC	GGTCCTGTCCTGTCCAAATC	82
CD44v9	AGCAGAGTAATTCTCAGAC	TGATGTCAGAGTAGAAGTTGTT	86
CD44v10	CCTCTCATTACCCACACACG	CAGTAACTCCAAAGGACCCA	81
Beta-actin	GTGAAGGTGACAGCAGTCGGTT	GAAGTGGGGTGGCTTTTAGGAT	157
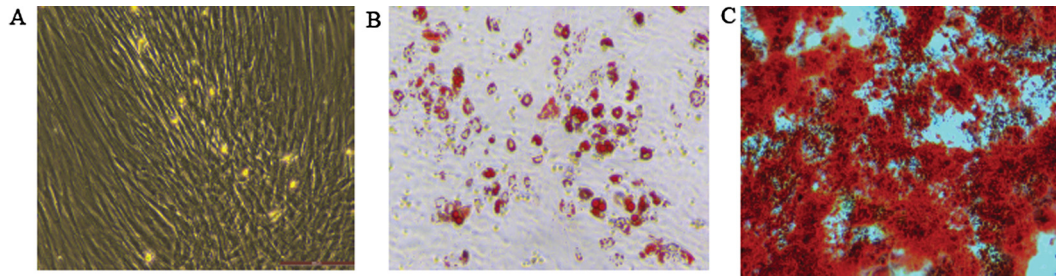
Fig. 1. Morphologies (micrographs) of hBMSCs differentiated into adipocytes and osteocytes. A: Morphologies of hBMSCs at passage 4 (100×); B: hBMSCs differentiated into adipocytes (Oil red O staining) (100×); C: hBMSCs differentiated into osteocytes (Alizarin Red staining) (100×).
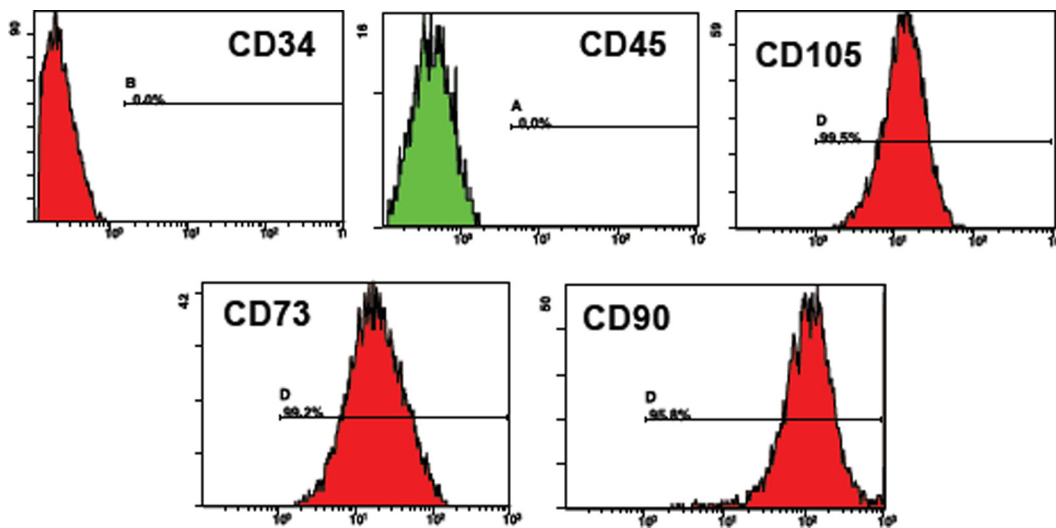

Fig. 2. Flow cytometry analysis of cell surface markers expressed on hBMSCs at passage 4.

of the multipotent differentiation potential of hBMSCs showed that Oil red-O positive fat cells were generated from hBMSCs after adipogenic induction for 18 days (Fig. 1B); whereas for the differentiation of osteoblasts, Alizarin Red positive cells were visualized after osteogenic induction for 21 days (Fig. 1C).

3.2. Immunophenotyping of hBMSCs

The passage 4 of hBMSCs expressed CD73 (99.2%), CD105 (99.5%), and CD90 (95.8%), but the surface markers CD45 and CD34 were almost not present (Fig. 2). Results indicated that the

cultured cells showed expressions of markers of mesenchymal cells, but not hematopoietic cells.

3.3. The expression levels of CD44S and CD44V

We examined the expression levels of CD44S and CD44V in the four hBMSC samples using q-PCR. Amplification curves of CD44S and CD44V showed both CD44S and CD44V were expressed at a certain level but the expressions of CD44V2 and CD44V5 were lower among these variants (Fig. 3). Analysis of the Cp values of the amplification curves showed that the expression of CD44S was significantly higher than the expression of each CD44V in
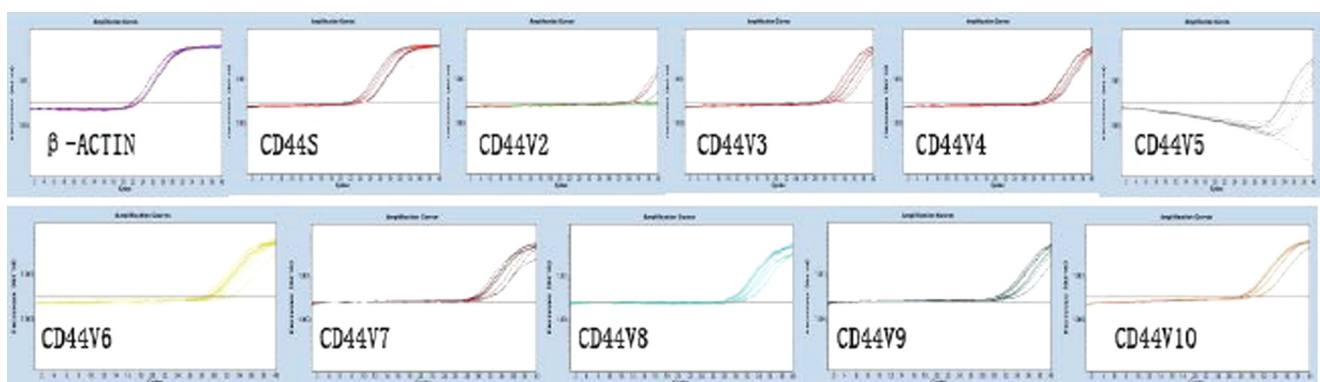

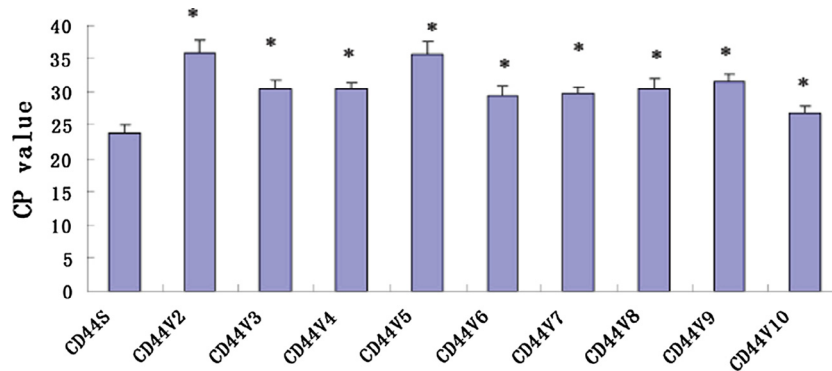**Fig. 3.** q-PCR amplification curves of CD44S and CD44 variants.

Fig. 4. Mean Cp values of amplification curves for CD44S and CD44V (n = 4). *When compared with CD44S: $p < 0.05$.
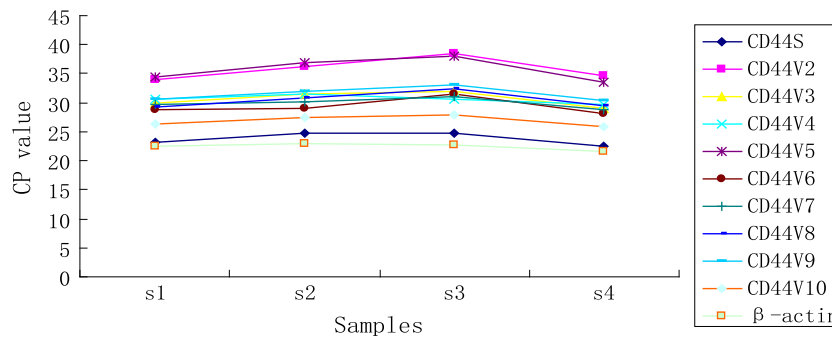

Fig. 5. Mean Cp values of amplification curves for CD44S and CD44V in the sample 1.

different hBMSCs (Fig.4). By calculating the average Cp value for the four samples, the mean Cp of CD44S was about 23.85, with the mean Cp of CD44V10 being the highest at 26.89 This suggested that the expression of CD44S was at least 10 times as high as the other CD44V variants in hBMSCs . In the sample 1, CD44S expression was also higher the other CD44V (Fig. 5),and in other samples have same result .These results indicated there were significant differences in the distributions of CD44S and CD44V gene expressions in hBMSCs, and CD44S expression was dominant.

4. Conclusion

In terms of the molecular mechanism, the CD44 molecule was required for the migration of exogenous hBMSCs into damaged tissue. The results of this study showed significant differences in the distribution of CD44S and CD44V gene expressions in hBMCS, demonstrating that CD44S expression was dominant. This suggested that if the expression pattern of CD44 was to be affected by different factors, it may impact the migration ability of hBMSCs.

Acknowledgements

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