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Evidences for correlation between the reduced VCAM-1 expression and hyaluronan synthesis during cellular senescence of human mesenchymal stem cells

Eun Mi Jung [a], Ohsuk Kwon [a], Ki-Sun Kwon [b], Yee Sook Cho [c], Sang Ki Rhee [e], Jeong-Ki Min [d,*], Doo-Byoung Oh [a,*]

[a] Integrative Omics Research Center, Korea Research Institute of Bioscience & Biotechnology (KRIBB), 111 Gwahangno, Yuseong-gu, Daejeon 305-806, Republic of Korea
[b] Aging Research Center, Korea Research Institute of Bioscience & Biotechnology (KRIBB), 111 Gwahangno, Yuseong-gu, Daejeon 305-806, Republic of Korea
[c] Development and Differentiation Research Center, Korea Research Institute of Bioscience & Biotechnology (KRIBB), 111 Gwahangno, Yuseong-gu, Daejeon 305-806, Republic of Korea
[d] Antibody Therapeutics Research Center, Korea Research Institute of Bioscience & Biotechnology (KRIBB), 111 Gwahangno, Yuseong-gu, Daejeon 305-806, Republic of Korea
[e] Department of Medical Biotechnology, Soonchunhyang University, Asan, Chungnam 336-745, Republic of Korea

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ABSTRACT

Mesenchymal stem cells (MSCs) undergo cellular senescence during in vitro expansion culture, which accompanies the loss of migration and homing abilities. In this study, we analyzed expression levels of several surface markers of human MSCs at different passages of expansion culture. It has been shown that expression of vascular cell adhesion molecule-1 (VCAM-1) was most markedly decreased among the tested markers in the senescent MSCs. Interestingly the reduced VCAM-1 expression could be restored by applying hyaluronan, a major glycosaminoglycan ligand of CD44, to the culture. It was found that the hyaluronan level in extracellular and pericellular matrices was greatly reduced in the senescent MSCs, mainly due to the decreased expression of hyaluronan synthases, suggesting a correlation between the reduced VCAM-1 expression and hyaluronan synthesis. In fact, when hyaluronan synthases were knock-downed by siRNA transfection, the VCAM-1 expression was also reduced. Our results indicate that VCAM-1 expression in the senescent MSCs was down-regulated because of the reduced synthesis of hyaluronan. Thus, we suggest that hyaluronan supplementation in expansion culture of MSCs would compensate adverse effects induced by its decreased synthesis and subsequently enhance cell adhesion and migration abilities.

1. Introduction

Mesenchymal stem cells (MSCs) are adult stem cell populations possessing the potential to differentiate into mesoderm-type cells such as osteoblasts, chondrocytes and adipocytes. MSCs are also able to home to the sites of inflammation and injury contributing to wound healing. Thus, they have been highlighted as a promising tool for cellular therapy and tissue engineering [1]. MSCs can be easily isolated from various tissues including bone marrow, peripheral blood and adipose tissues. However, their population is so low that isolated MSCs have to be cultured in vitro to obtain sufficient cell numbers for clinical uses. Unfortunately, during this long-term expansion process, MSCs experience cellular senescence, gradually losing their capabilities for differentiation and proliferation until their cell division is eventually arrested. In addition, it has been also reported that MSCs lost their homing ability during in vitro expansion culture [2].

Unlike hematopoietic stem cell which can be identified by its unique surface marker, CD34, MSCs were currently identified by a panel of positive and negative markers. Several CD antigens including CD44, CD73, CD90, CD105 and CD106 (VCAM-1) have been used as positive markers to discriminate human MSCs while negative markers used to detect contaminated hematopoietic cells [1]. Several groups have analyzed the changes of surface marker expressions caused by prolonged cultivation and reported the attenuated expressions of several markers including VCAM-1 and intercellular adhesion molecule (ICAM)-1 [3,4]. Cell adhesion molecules such as VCAM-1 and ICAM-1 have been known to mediate the interaction of MSCs with endothelial cells, which is essential for homing ability of MSC. Therefore, the reduction of VCAM-1 expression during the expansion culture might be related with decreased homing ability of the senescent MSCs. Recently, CD44 has been reported to participate in the homing process by interacting with its glycosaminoglycan ligand, hyaluronan and promote

* Corresponding authors. Fax: +82 42 860 4137 (J.-K. Min), +82 42 860 4594 (D.-B. Oh).
E-mail addresses: jekmin@kribb.re.kr (J.-K. Min), dboh@kribb.re.kr (D.-B. Oh).

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adhesion and migration of MSCs [5]. It has been suggested that hyaluronan–CD44 interaction plays critical roles in initiating intracellular signal-mediated events, including proliferation, cell adhesion and migration in other types of cells. It was also reported that hyaluronan–CD44 interaction induced VCAM-1 expression in three types of cells, kidney tubular epithelial cells, rheumatoid synovial cells and osteoblasts [6–8]. However, the relation between hyaluronan–CD44 interaction and VCAM-1 expression has not been studied yet in MSCs although it seems crucial to understand reduced capabilities of cell adhesion and migration in the senescent MSCs.

In the present study, we examined changes of expression of surface markers including VCAM-1 and hyaluronan level in extracellular and pericellular matrices during long-term expansion culture of human MSCs. Then, it was found that decrease of VCAM-1 expression in the senescent MSCs was caused by reduced synthesis of hyaluronan. Furthermore, it was shown that supplementation of hyaluronan in the expansion culture could induce expression of VCAM-1, suggesting the possibility to employ hyaluronan to enhance cell adhesion and migration abilities of the senescent MSCs.

2. Materials and methods

2.1. Reagents

Hyaluronan prepared from human umbilical cord and biotinylated hyaluronic acid binding protein (B-HABP) were purchased from Sigma and NORTH BioProducts, respectively. The antibodies (Abs) in human MSC marker antibody kit (R&D system) were used to examine the expressions of surface markers. Rabbit polyclonal anti-VCAM-1 Ab, anti-β-actin Ab and HRP-conjugated anti-rabbit IgG Ab were purchased from Santa Cruz. Alexa Fluro® 488-conjugated streptavidin, and Alexa Fluro® 555-conjugated anti-Mouse IgG Ab were from Invitrogen. All other reagents, unless specified, were molecular biological or analytical grade and purchased from Sigma.

2.2. Cell lines and culture conditions

Human MSCs purchased from Lonza were grown in Poietics® MSCGM™ Bullet Kit® (Lonza) in a 5% CO_2 incubator at 37 °C. Purchased MSCs were in passage 2 and plated in fresh culture flask. When MSCs reached 80% confluence, they were split 1:3 into new plate. For long-term culture, MSCs were passaged 12 times for ~2 months until passage number reached 14. For hyaluronan treatment, MSCs were seeded in 60 mm dish and cultured until the subconfluence. Then, these cells were incubated with 1–10 μg/ml hyaluronan in the same culture medium for 6 h at 37 °C as previously described [8]. Senescence-associated β-galactosidase (SA-β-gal) assay was performed according to a protocol described previously [9].

2.3. Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen). Four micrograms of total RNA was used for cDNA synthesis using M-MLV reverse transcriptase (Promega). The synthesized cDNA samples were used as templates for PCR with corresponding primers and the resulting PCR products were detected by agarose gel electrophoresis. Quantitative real-time PCR analyses were performed in a Rotor-gene 6000 thermocycler (Corbett Research) using 1× SYBR Green mix (Invitrogen). Specific primers for amplification of hyaluronan synthase genes, HAS1, HAS2 and HAS3 were synthesized by Bioneer as previously described [10]. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization.

2.4. Western blot analysis

Cell lysates (25 μg protein per lane) were subjected to SDS–PAGE and transferred to PVDF membranes. The membranes were incubated with the indicated primary Ab followed by secondary Ab. Target protein bands were visualized using chemiluminescent substrates (GE Life Sciences).

2.5. Flow cytometry and immunofluorescence microscopy

For flow cytometric analysis, MSCs were incubated on ice with a primary antibody and then stained with FITC-conjugated secondary Ab. Stained cells were analyzed using FACSCalibur (BD Biosciences). For immunofluorescence microscopy, 3×10^5 MSCs were seeded in cover glass bottom dish and incubated in a 5% CO_2 incubator at 37 °C overnight. After fixed with 2% paraformaldehyde, MSCs were blocked in 2% BSA blocking solution and then incubated with anti-CD44 mAb and B-HABP at 4 °C. After PBS washing, they were labeled with Alexa Fluro® 555-conjugated secondary Ab and Alexa Fluro® 488-conjugated streptavidin. The image was visualized using an Olympus IX81-ZDC inverted fluorescence microscopy.

2.6. siRNA transfection

For knock-down of HAS1 gene, a mixture of four siRNAs (ON-TARGETplus SMARTpool siRNA) was purchased from Dharmacon. For HAS2, a single 21-bp siRNA (5′-UCAUGUCUCUCUACUCAGU-3′) with an overhang of dTdT was synthesized by Bioneer. Negative control siRNA was also purchased from Bioneer. MSCs were seeded into 60 mm dish and transfected with 200 pmol of siRNA using lipofectamine 2000 reagents (Invitrogen).

3. Results

3.1. Expression changes of human MSC markers during long-term expansion culture

In order to investigate the effect of long-term culture on expressions of surface markers, human MSCs were cultured up to the passage number 14 in commercial Lonza medium supplemented with 10% serum. Growth rate of MSCs was gradually decreased and their sizes became larger with irregular and flat morphology. When SA-β-gal assay was performed to detect senescent cells, majority of cell population in passage 14 was intensely stained (Supplementary Fig. 1S), indicating that these cells entered the status of senescence. Expressions of various surface markers in culture passage 5 and 14 were compared by flow cytometric analysis (Fig. 1A). Cell populations expressing negative markers (CD19 and CD45) were measured less than 2% in all cases (data not shown). Most of the tested positive MSC markers displayed attenuated expressions in passage 14 compared to those in passage 5 even though the degrees of reductions were variable (Fig. 1B). The most dramatic change was observed in the expression of CD106, known as VCAM-1. Its expression level decreased from 17% of total cell population in passage 5 to 2% in passage 14, which was almost 9-fold reduction. In contrast, the expression level of Stro-1 was almost same between passages 5 and 14. We also checked expression change of ICAM-1, which was shown to decrease during long-term culture of MSCs in the previous study [3]. However, in our hands, its expression did not change much or rather appeared to increase a little bit over the tested passages on the contrary to the previous study (data not shown).

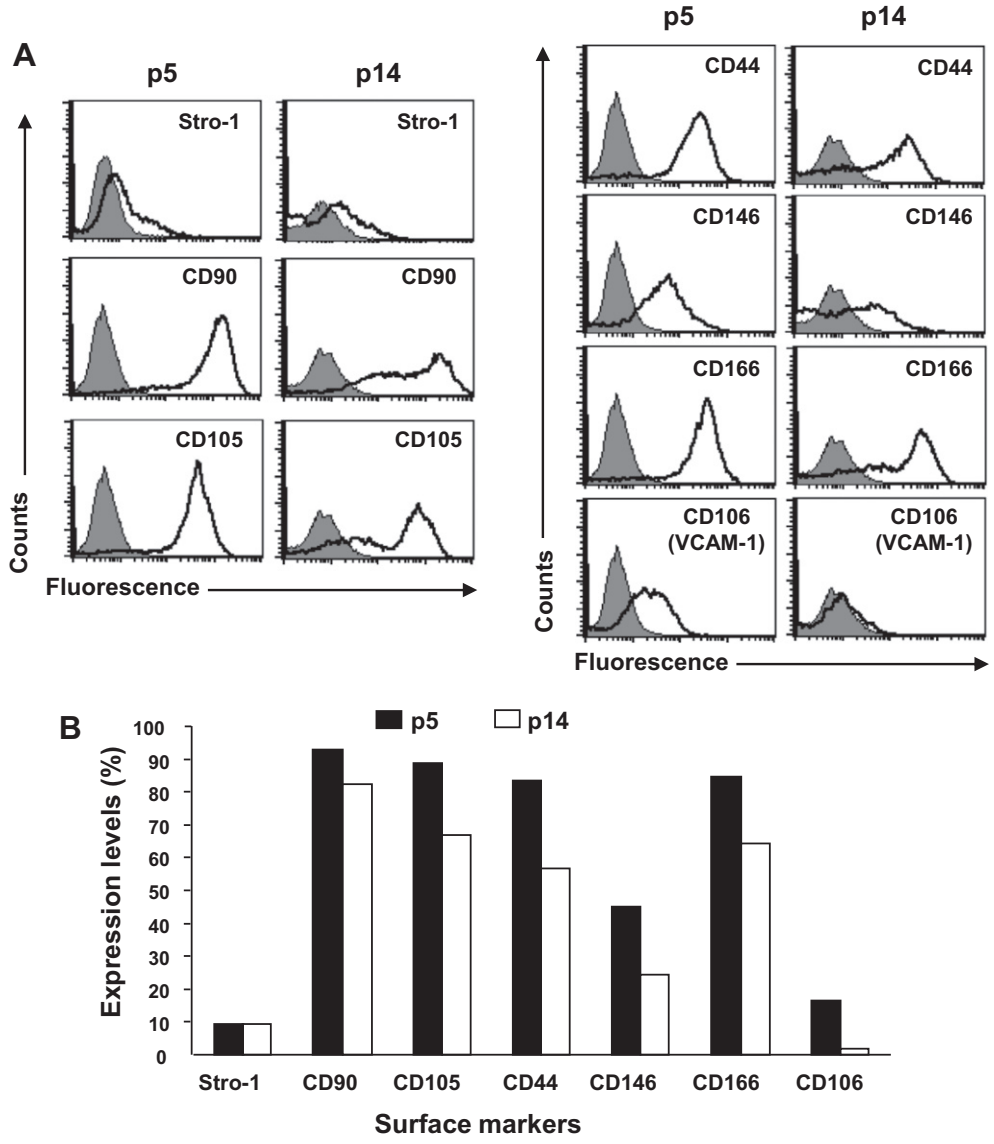

Fig. 1. Down-regulation of several surface markers in the senescent MSCs. (A) Expression of surface markers of MSC in the culture passage 5 (p5) and 14 (p14) were analyzed by flow cytometry. Shaded area of the histogram represents the profile of isotype control while un-shaded area indicates the cells stained with the antibody against the indicated marker. (B) Bar graphs representing the percentage of positive cell population expressing the indicated surface marker. Positive cell populations were calculated after setting the threshold line at the highest fluorescence value of each isotype control.

3.2. Hyaluronan treatment up-regulates VCAM-1 expression decreased in the senescent MSCs

Since it has been reported in three cell types (kidney tubular epithelial cells, rheumatoid synovial cells and osteoblasts) that treatment of hyaluronan resulted in up-regulation of VCAM-1 expression [6–8], we investigated the question of whether hyaluronan could also affect VCAM-1 expression in human MSCs. Hyaluronan was applied to MSC cultures in different passages and after 6 h of incubation the VCAM-1 expression was analyzed by both Western blot and flow cytometry (Fig. 2). Western blot allowed detection of total VCAM-1 existed in whole cell lysate whereas flow cytometric analysis only detected VCAM-1 displayed on the cell surface. Both analyses showed the same tendency of increased VCAM-1 expression upon hyaluronan treatment. However, we noticed that larger amount of hyaluronan was required to observe the full recovery of VCAM-1 expression in flow cytometry than Western blot analysis. This insensitivity of flow cytometric analysis could be due to the trapping of newly synthesized VCAM-1 in passing from ER to Golgi before emerging on plasma membrane. Stimulatory effects by hyaluronan treatment on VCAM-1 expression were seen in all passages tested, but the highest folds change was observed in passage 13.

To test the possibility of inflammation reaction by contaminating lipopolysaccharide (LPS) in hyaluronan preparation, endotoxin test was carried out. The endotoxin level in 10 μg/ml hyaluronan solution was estimated to be about 800 fg/ml, far less than the previously reported threshold amount (<1 ng/ml LPS) not affecting VCAM-1 expression [6]. Taken together, it was concluded that VCAM-1 expression was enhanced by hyaluronan itself but not by contaminating endotoxin.

To check the involvement of CD44 in up-regulation of VCAM-1 expression by hyaluronan, we examined whether CD44 cross-linking could induce similar effects on VCAM-1 expression of the senescent MSCs. Cross-linking of CD44 located on the cell surface could be achieved using a primary anti-CD44 mAb treatment followed by a secondary cross-linker reagent (anti-Fc Ab) treatment. It has been suggested that CD44 cross-linking mimics clustering of

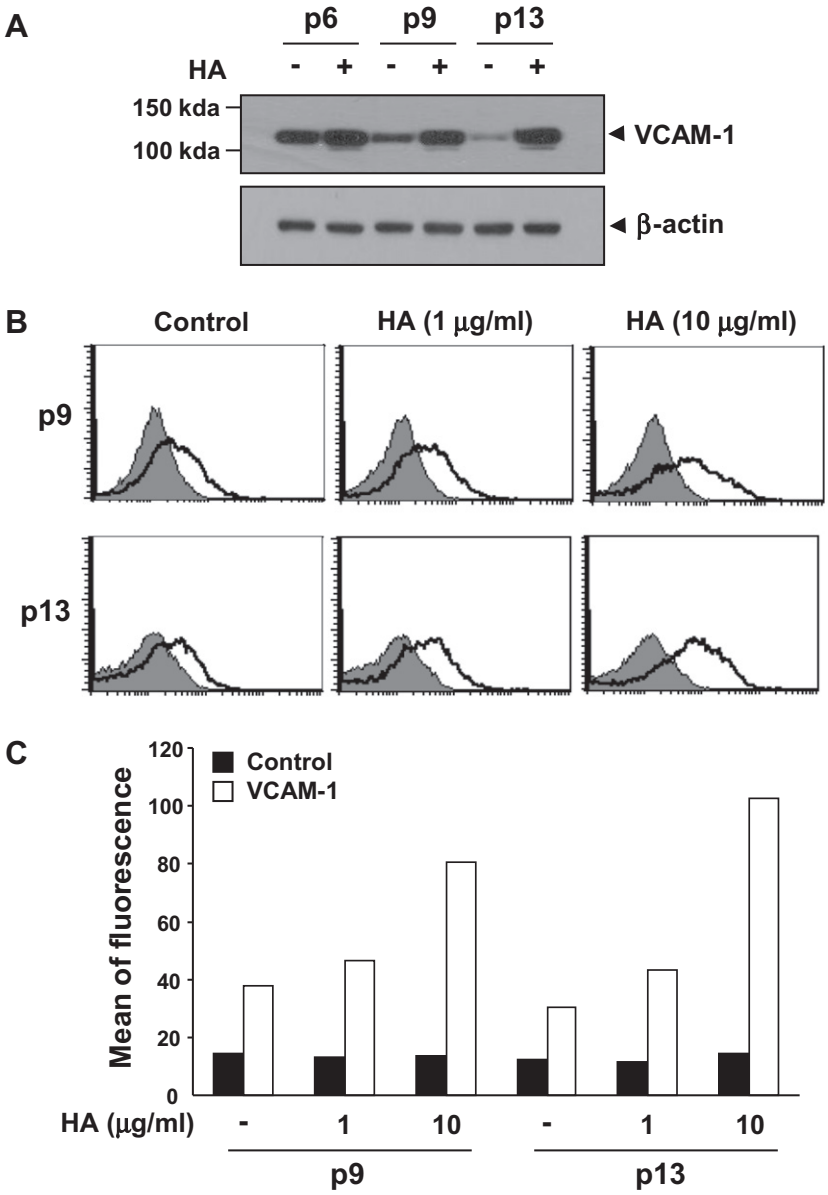

Fig. 2. Up-regulation of VCAM-1 expression by hyaluronan treatment. (A) VCAM-1 expression was detected by Western blot analysis from total cell lysates of MSCs in culture passage 6 (p6), 9 (p9) and 13 (p13) with or without addition of 1 μg/ml hyaluronan for 6 h. (B) Surface expression of VCAM-1 was analyzed by flow cytometry after the incubation with 1, or 10 μg/ml hyaluronan for 6 h. (C) Bar graphs representing the increases in mean fluorescent intensity induced by hyaluronan treatment.

CD44 induced by hyaluronan binding, which could transduce a signal into the cells for proliferation, adhesion and migration [7,8,11]. We observed that CD44 cross-linking significantly restored VCAM-1 expression in the senescent MSCs although the increased level of expression was much smaller than that by hyaluronan treatment (Supplementary Fig. 2S). On the whole, this result indicated that hyaluronan–CD44 interaction contributes to enhanced expression of VCAM-1 by hyaluronan treatment.

3.3. Hyaluronan synthesis decreases in the senescent MSCs

Since VCAM-1 expression, which could be regulated by hyaluronan, was markedly reduced in the senescent MSCs, we were interested in the change of hyaluronan level during in vitro expansion culture. Thus, hyaluronan level in extracellular and pericellular matrices was determined in early and senescent passages by immunofluorescence microscopy. After the fixation, MSCs were double-labeled with anti-CD44 Ab and biotinylated hyaluronan binding protein and then stained with fluorescence dye-labeled secondary Ab and streptavidin. As shown in Fig. 3A, the green fluorescence indicate hyaluronan in extracellular and pericellular matrices, which could be discriminated from red fluorescence displaying the boundary of plasma membrane stained by anti-CD44 Ab. It has been clearly observed that hyaluronan level was also drastically decreased during the expansion culture.

To investigate the reason for reduced hyaluronan level, the expression changes of three hyaluronan synthase genes (HAS1, HAS2 and HAS3) in passages 6, 9 and 13 were analyzed by RT-PCR. We observed that RT-PCR products of HAS1, HAS2 and HAS3 were clearly detected in early passages and vanished in a senescent passage although the bands corresponding to HAS3 displayed faint appearances among nonspecific bands (Fig. 3B). Quantitative real-time PCR results also indicated that expressions of hyaluronan synthase genes in MSCs greatly decreased as passage number increased (Fig. 3C). The amounts of the transcripts in passage 13 were only less than 20% of those in passage 6. When the relative

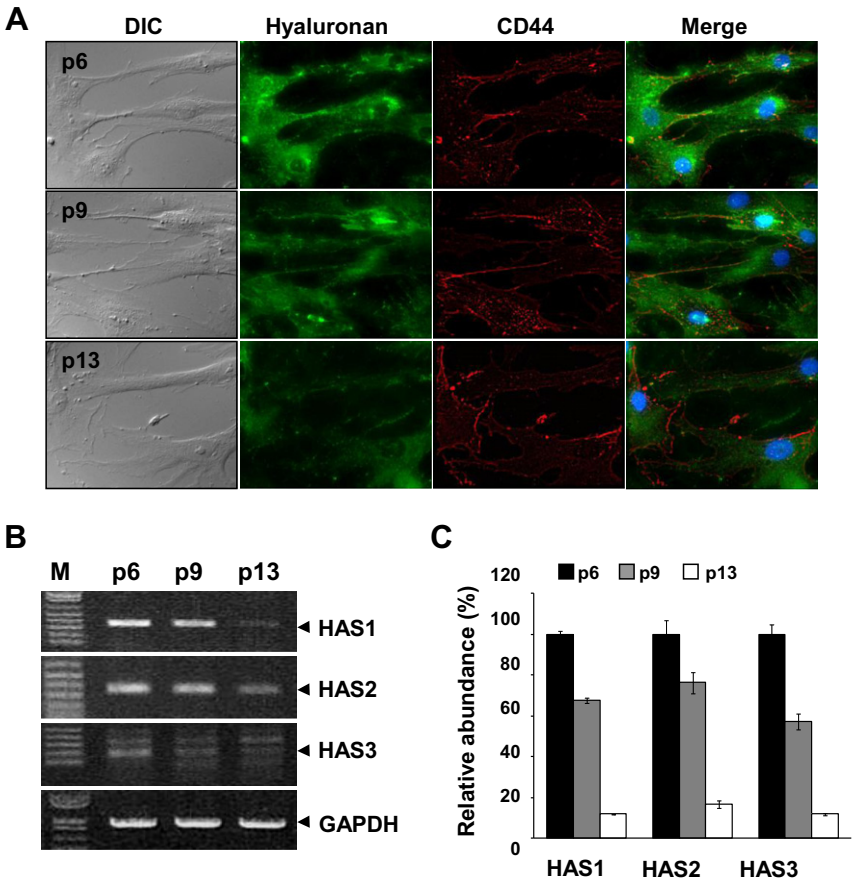

Fig. 3. Decreased synthesis of hyaluronan in the senescent MSCs. (A) Immunofluorescence images of hyaluronan in extracellular and pericellular matrices. Hyaluronan is represented by green fluorescence while red and blue fluorescence indicate plasma membrane stained by anti-CD44 Ab and nucleus, respectively. (B) Expressions of hyaluronan synthase genes (HAS1, HAS2 and HAS3) in MSCs of p6, p9 and p13 were analyzed by semiquantitative RT-PCR. (C) Culture passage dependent expressions of HASs were quantified by real-time PCR. The relative abundances of each transcript were normalized by setting those from p6 as 100%.

abundances of HAS1, HAS2 and HAS3 transcripts were calculated from real-time PCR results, HAS2 transcripts were turned out dominant as twice of HAS1 transcripts. In contrast, the level of HAS3 transcript was ~100-fold lower than that of HAS2. The expression of major hyaluronidase genes (HYAL1 and HYAL2) were also examined by real-time PCR but their expressions were relatively invariable over the tested passages (data not shown). Taken together, the above results indicate that hyaluronan level in extracellular and pericellular matrices decreases in senescent passages mainly due to down-regulated expression of hyaluronan synthase genes and thereby resulting in reduction of VCAM-1 expression.

3.4. Correlation between the reduced expressions of hyaluronan synthases and VCAM-1

In order to confirm the direct correlation between expression of hyaluronan synthases and VCAM-1, we determined the effects of knock-down of major hyaluronan synthases, HAS1 and HAS2 genes in passage 6 of MSCs by siRNA transfection (Fig. 4). Transfection of HAS1 siRNAs reduced 81% of HAS1 expression as well as 40% of HAS2 expression. On the other hand, HAS2 siRNA transfection specifically inhibited 71% of HAS2 expression. The combined transfection of HAS1 and HAS2 siRNAs decreased 73% and 74% of HAS1 and HAS2 expression, respectively. Western blot analysis also showed that VCAM-1 expression decreased significantly upon transfection of HAS1, HAS2 or combined siRNAs (Fig. 4C). Especially, combined siRNA treatment resulted in the most severe reduction of VCAM-1 expression, suggesting the additive effect of both HAS1 and HAS2 knock-down.

4. Discussion

Our results clearly showed that the reduced expression of VCAM-1 in the senescent human MSCs is correlated with the reduced level of hyaluronan. Hyaluronan comprising repeating units of glucuronic acid and *N*-acetylglucosamine is directly synthesized by hyaluronan synthases located on the plasma membrane and degraded by hyaluronidase. Here, we demonstrated that expression of hyaluronan synthases greatly decreased in the senescent human MSCs (~5-fold decrease in passage 13 compared to passage 6). In contrast, no significant changes in the expression of major hyaluronidases (HYAL1 and HYAL2) were observed in the senescent MSCs. Therefore, the significant reduction of hyaluronan level in the senescent MSCs observed by immunofluorescence microscopy would result from the decreased expressions of hyaluronan synthases.

In the present study, we showed that both hyaluronan treatment and CD44 cross-linking could induce up-regulation of VCAM-1 expression in the senescent MSCs. However, the increased level of VCAM-1 expression induced by CD44 cross-linking was much smaller than that by hyaluronan treatment. Thus, there remained the possibility that hyaluronan increased VCAM-1 expression through multiple signal transduction mechanisms including CD44-dependent and -independent pathways. Hyaluronan signaling

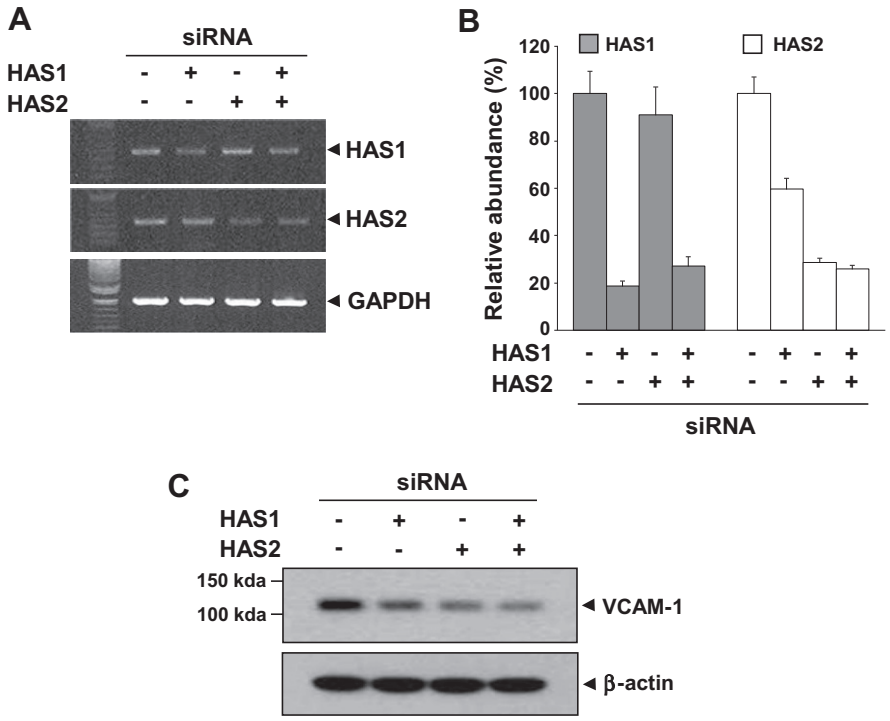

Fig. 4. Knock-down of hyaluronan synthases leads to decreased expression of VCAM-1. Expressions of HAS1 and HAS2 mRNA in MSCs of passage 6 were examined by RT-PCR (A) and quantitative real-time PCR (B) after the transfection of control, HAS1, HAS2 and combined (HAS1 and HAS2) siRNAs. (C) VCAM-1 expression in the siRNA-transfected MSCs was analyzed by Western blot.

has been known to occur even in the absence of CD44 [12,13]. Especially, a receptor for hyaluronan-mediated motility (RHAMM) has been shown to compensate for the loss of CD44, supporting cell migration and up-regulation of hyaluronan inducible genes [13]. Another hyaluronan receptor, layilin was also reported [14]. It should be noted that the finding of this study is restricted to the contribution of hyaluronan–CD44 interaction to increased expression of VCAM-1 in the senescent MSCs. It still remains to be elucidated whether other signaling pathways are involved.

It has been reported that constitutive interactions between hyaluronan and CD44 could promote the signaling for cell proliferation, which can be reversed if constitutive hyaluronan–CD44 interactions are inhibited [15,16]. We observed that reduced expression of hyaluronan synthases resulted in dramatic decrease of hyaluronan level in extracellular and pericellular matrices in the senescent MSCs. Consequently, this decrease would weaken or disturb normal hyaluronan–CD44 interaction, which will lead to reduction of intracellular signaling in turn. Our result showed that VCAM-1 expression was also under the regulation of signaling cascade induced by hyaluronan–CD44 interaction. Taken together, it can be concluded that the decreased expression of VCAM-1 in the senescent MSCs was caused by reduced synthesis of hyaluronan.

In vitro expansion culture of MSCs is an inevitable process because the number of cells isolated from human tissues is often limited. However, it always accompanies cellular senescence of MSCs, which has been known to lose migration and homing ability [2]. Our finding about decreased expressions of VCAM-1 and hyaluronan synthases in the senescent MSCs could partly explain this reduced migration and homing ability. Hyaluronan interacts with its receptor CD44, which plays important roles in migration of MSC [5]. Also, adhesion molecules including VCAM-1 were found to participate in rolling and adhesion of MSCs. Therefore, the decreased expressions of these major players in adhesion and migration will lead to reduced homing ability of the senescent MSCs. Further, this speculation would suggest promising applications of hyaluronan in cell therapy using MSCs. Hyaluronan supplementation in expansion culture of MSCs would compensate the adverse effects induced by decreased synthesis of hyaluronan and therefore contribute to increasing migration and homing abilities which are important for wound healing effect of MSCs. Our next goal is to determine whether the senescent MSCs expanded in hyaluronan-supplemented culture can have better proliferation rate, homing ability and wound healing effect in mouse model.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.12.003.

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