Identification of CD44 as a senescence-induced cell adhesion gene responsible for the enhanced monocyte recruitment to senescent endothelial cells

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Mun GI, Boo YC. Identification of CD44 as a senescence-induced cell adhesion gene responsible for the enhanced monocyte recruitment to senescent endothelial cells. Am J Physiol Heart Circ Physiol 298: H2102–H2111, 2010. First published April 9, 2010; doi:10.1152/ajpheart.00835.2009.—The mechanism that is responsible for progression of atherosclerosis seen with increasing age remains controversial. This issue was addressed in the present study, by searching for genes that are uniquely expressed in senescent endothelial cells and functionally involved in inflammatory leukocyte adhesion recognized as a critical step in the initiation of atherogenesis. Senescent human umbilical vein endothelial cells (HUVECs) prepared by continuous subcuturing in vitro showed higher binding affinity for monocytes (THP-1 cells, human acute monocytic leukemia cell line) compared with young cells. Gene expression profiles between young and senescent endothelial cells were compared by the cDNA microarray method, and CD44 was identified as one of the “senescence-induced cell adhesion genes” whose expression was upregulated in senescent cells and whose gene ontology annotation indicated their role in cell adhesion. The enhanced gene expression of CD44 in senescent endothelial cells was verified both at the mRNA and protein levels. Adhesion of monocytes to senescent endothelial cells was significantly reduced following pretreatment of endothelial cells with the CD44 antibody or small-interfering RNA, thus reinforcing the critical role of CD44 in the inflammatory event. Exogenous expression of CD44 in young HUVECs and in human aortic endothelial cells led to an increase in monocyte adhesion. CD44 expression levels in the rat aorta endothelium were found to increase in an age-dependent manner, as determined by immunohistochemistry and Western blotting. CD44 and other senescence-induced cell adhesion genes identified in this study may provide the novel targets for the prevention of inflammatory leukocyte adhesion leading to the development atherosclerosis.

atherosclerosis; endothelial cells; cellular senescence; monocytes adhesion; CD44

ATHEROSCLEROSIS DENOTES THE accumulation of lipids and fibrous elements in the intimal lining of the large- and medium-sized arteries. Its development takes place in an insidious manner step-by-step involving endothelial cell injury, migration of inflammatory cells, deposition of lipid and proliferation of smooth muscle cells, growth of this mass (atheroma) into the vessel lumen, and rupture of the plaque with subsequent thrombosis (22). Therefore, the structural and functional integrity of the endothelium, which consists of a single layer of endothelial cells, is critical for the prevention of atherosclerosis and cardiovascular disease from the beginning (10).

Normal function of the endothelium includes the regulation of vasodilatation, coagulation, inflammation, and immune response. Endothelium also acts as a physical barrier that protects subendothelial cells from interacting with the cells in blood. However, under certain pathological conditions, various inflammatory stimuli activate the endothelial cells to express selective adhesion molecules that results in binding of monocytes and other inflammatory cells, leading to the initiation of the atherosclerotic lesion formation (30). Various cardiovascular risk factors, including elevated levels of low-density lipoprotein, smoking, hypertension, and immune reaction, are known to induce endothelial cell activation (12, 13). Aging is an additional risk factor closely associated with the incidence of atherosclerosis, but the underlying mechanism is not well understood, probably because of multifarious changes of vascular microenvironments (16).

After a finite number of replications, endothelial cells, like other somatic cells, enter a state of replicative senescence that is accompanied by morphological changes and functional degeneration (9). Therefore, endothelial cell senescence has been deemed as a potential mechanism for age-related increase in cardiovascular diseases as well as an important manifestation of human aging (25). In fact, senescent endothelial cells have been detected at atherosclerotic lesions and in vessels of elderly people (3, 14, 33). Previous studies have demonstrated that adhesion potential of inflammatory leukocytes to endothelial cells progressively increases with the age of endothelial cells (24). Therefore, it could be hypothesized that endothelial cells may express higher levels of certain adhesion molecules as a result of cellular senescence, providing vulnerable microenvironments for the initiation of inflammatory atherogenesis.

The purpose of the present study was to understand the mechanism for enhanced adhesion of leukocytes to senescent endothelial cells. By assuming the presence of potential adhesion molecules uniquely expressed at a high level in senescent endothelial cells, differential gene expression of young and senescent endothelial cells cultured under static and laminar shear stress (LSS) conditions were examined by the cDNA microarray method. The LSS conditions were included because endothelial cells in blood vessels are actually exposed to blood flow, which regulates a variety of endothelial functions and gene expressions in a magnitude- and flow pattern-dependent fashion (6, 21, 31). The present study identified the “senescence-induced cell adhesion genes” whose expression was upregulated in senescent cells compared with younger cells, irrespective of culture conditions. Among these genes, CD44, a well-known cell adhesion molecule, was chosen for further studies, examining its potential as a key molecule responsible for the enhanced leukocyte adhesion to senescent endothelial cells.
MATERIALS AND METHODS

Preparation and characterization of senescent human umbilical vein endothelial cells. Human umbilical vein endothelial cells (HUVECs) obtained from Clonetics Cambrex (Rockland, ME) were cultured in EBM-2 medium containing endothelial growth supplements (Clonetics Cambrex), 10% FBS (GIBCO-BRL, Grand Island, NY), and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) on 0.2% gelatin-coated 100-mm tissue culture dishes (BD Biosciences, San Jose, CA) at 37°C and 5% CO₂ (26). Cells were serially subcultured at ratios of 1 to 3 until they evidenced senescence-associated phenotypes (27). Cell morphology was evaluated under an Eclipse TS100 inverted phase microscope from Nikon (Melville, NY). Cytochemical staining of young (P4) and senescent (P18) cells for senescence-associated β-galactosidase (SA-β-gal) was conducted as previously described (35). Total β-galactosidase activity in the cell lysates was assayed using a β-galactosidase assay kit from Stratagene (LaJolla, CA). The protein contents of the cell lysates were determined using a DC protein assay kit (Bio-Rad, Hercules, CA).

Cultivation of human aortic endothelial cells. Human aortic endothelial cells (HAECs) were purchased from Cascade Biologics (Portland, OR) and cultured in Medium 200 with low serum growth supplements (Cascade Biologics) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) on 0.2% gelatin-coated 100-mm tissue culture dishes (BD Biosciences) at 37°C and 5% CO₂.

Cultivation and fluorescence labeling of THP-1 cells. THP-1 cells (human acute monocytic leukemia cell line) obtained from the Korea Cell Line Bank (Seoul, Korea) were cultured in RPMI 1640 medium (GIBCO-BRL) containing FBS (10%), antibiotics, and β-mercaptoethanol (0.05 mM) in T-25 flasks (Nunc, Roskilde, Denmark) in an upright position. For fluorescence labeling, monocytes were washed with PBS two times and suspended at 5 × 10⁶ cells/ml in PBS containing 5 μg/ml 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxyethyl ester (Molecular Probes, Carlsbad, CA), followed by incubation at 37°C for 45 min. The labeled cells were then collected, washed with PBS two times, and suspended in RPMI medium to be added to endothelial cell culture.

Cell adhesion assay. Young and senescent HUVECs were seeded on 24-well tissue culture plates at 2–4 × 10⁴ cells/cm² and cultured in EGM-2 medium for 2 days. HUVECs were either stimulated with 4 ng/ml tumor necrosis factor-α (TNF-α) for 4 h or treated with a vehicle. In some experiments, cells were pretreated with an antibody in combination with TNF-α. Next, culture medium of HUVECs was replaced by RPMI medium. To the HUVECs culture, the fluorescence-labeled THP-1 cells were added at a 1:1 ratio. After coincubation of HUVECs and THP-1 cells in RPMI medium for 1 h, nonadherent THP-1 cells were removed by washing two times with PBS, with caution taken not to disturb the endothelial cell monolayer. Fresh RPMI medium was supplied to the remaining cells on the culture plates. Florescence-labeled THP-1 cells adhering to the endothelial cell surface were observed with a Nikon eclipse TE2000-U micro-

Fig. 1. Endothelial cell senescence promotes monocyte adhesion. A: human umbilical vein endothelial cells (HUVECs) at different passage numbers (P4 and P18) were stained for senescence-associated β–galactosidase activity at pH 6.0. B: total β–galactosidase (β-gal) activity of cell lysates was determined and normalized for protein amount. C: monocytes (THP-1 cells) were coincubated for 1 h with young and senescent HUVECs pretreated with 4 ng/ml tumor necrosis factor-α (TNF-α) or vehicle for 4 h. Fluorescing monocytes that adhered on the endothelial cell surface were observed under a fluorescence microscope. D: fluorescence intensity was determined and normalized/no. of endothelial cells to quantitatively estimate monocyte adhesion. Fold changes are over young cells treated with vehicle only. Data are expressed as means ± SE (n = 3 experiments). Bars not sharing the same letter differ significantly from each other (P < 0.05).
senescence. For quantification, adherent cells were lysed in 200 μl of 0.1 M Tris containing 0.1% Triton X-100 and centrifuged at 13,000 rpm for 15 min. The fluorescence intensity of supernatants was determined at the excitation wavelength of 485 nm and emission wavelength of 535 nm, using an LS 55 Fluorescence Spectrometer (Perkin Elmer Instruments, Waltham, MA), and normalized for number of endothelial cells.

LSS treatments of endothelial cells and gene expression profiling. Young or senescent HUVECs grown on 100-mm culture dishes were exposed to steady LSS at 12 dyn/cm for 24 h or maintained under static conditions. LSS was provided using a rotating Teflon cone (0.5° cone angle) mounted on a culture dish, as previously described (2, 32). Total cellular RNA was extracted from cells using the RNeasy kit (Qiagen, Valencia, CA), and gene expression profiles were analyzed on a GeneChip HG-U133 Plus 2.0 (Affymetrix, Santa Clara, CA) (27). Three chips were used for each condition. The complete datasets have been deposited in the Gene Expression Omnibus database (accession no. GSE13712). Each of these experimental samples was compared with each of the control samples, resulting in nine pairwise comparisons. This approach, which is based on the Welch’s t-test, provided a P value for each gene expression change. Expression of a gene was considered to be altered if the P values for the average fold changes were <0.05.

RT-PCR. RT-PCR was conducted using the GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) in a reaction mixture (20 μl) containing Maxime RT-PCR PreMix (NtRON Biotechnology, Seongnam, Korea), 250 ng RNA, and 10 pmol of gene-specific primer sets (Bioneer, Daejeon, Korea). The sequences of PCR primers were as follows: alternatively spliced transcripts of human CD44 gene (GeneBank accession no., variant 1, NM000610.3; variant 2, NM001001389.1; variant 3, NM001001390.1; variant 4, NM001001391.1) 5′-GAG-CCA ATT ACC ATA ACT ATT G-3′ (sense) and 5′-GCA AAT TGC AAG AATCAA-3′ (antisense); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GeneBank accession no. NM002046) 5′-GCC AAA AGG GUC GUC=5′-GAA CAA GGA GUC GUC (antisense). The PCR products were electrophoresed in a 1.0% agarose gel with a 100-bp Plus DNA ladder (Bioneer) as a size marker. The gel was ethidium bromide-stained, and the band intensities were determined using a Gel Doc system (Bio-Rad).

Western blotting. Western blotting of the cell lysates or tissue homogenates was conducted as previously described (19). Cells were lysed in a lysis buffer (10 mM Tris-Cl, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% Triton X-100, and 1% deoxycholate, pH 7.2) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Roche, Mannheim, Germany). Tissues were homogenized in a buffer containing 50 mM Tris, 10% glycerol, 140 mM NaCl, 1% Triton X-100, and the following supplements: 5.5 μM leupeptin, 5.5 μM pepstatin, 20 KIU/ml aprotinin, 2 mM Na3VO4, 1 mM NaF, 100 μM ZnCl2, 20 mM β-glycerophosphate, and 20 μM PMSF. Cell lysates or tissue homogenates were centrifuged at 13,000 rpm for 15 min, and the supernatants were collected. Proteins were denatured in Laemmli sample buffer for 5 min at 95°C, resolved on 10% SDS-PAGE gel, and transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia, Little Chalfont, UK). The membranes were incubated overnight with a primary antibody at 4°C and then with a secondary antibody conjugated to horseradish peroxidase (Cell Signaling, Danvers, MA) for 1 h at room temperature. The immunoreactive bands were detected using an ECL kit (Amersham Pharmacia) and analyzed using the NIH Image program. Goat polyclonal antibody generated against the NH2-terminal region of human CD44 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Nonimmune IgG and monoclonal β-actin antibody were from Sigma-Aldrich (St. Louis, MO).

Immunocytochemistry. Young and senescent HUVECs were fixed with 2.5% paraformaldehyde in PBS for 30 min and blocked with 5% donkey serum (Jackson ImmunoResearch Laboratories, Bar Harbor, ME) in PBS containing 0.3% Triton X-100 and 0.1% BSA for 1 h. Cells were incubated with goat polyclonal CD44 antibody followed by 1 h incubation with a secondary antibody (fluorescein isothiocyanate-conjugated donkey anti-goat IgG antibody) diluted 1:100 in PBS. After being rinsed, cells were counterstained with 4′,6-diamidino-2-phenylindole nuclear stain. The slides were mounted under glass cover slips and examined with a confocal microscope (LSM 5 PASCAL; Carl Zeiss, Oberkochen, Germany).

Small-interfering RNA transfection. Human CD44 small-interfering RNA (siRNA)-1 (no. 1299001, HSS190457) with nucleotide sequences in the coding region of human CD44 gene transcript variants 1–4 and a negative control oligoribonucleotide duplex (no. 12935300) with scrambled sequences were obtained from Invitrogen (Invitrogen, Grand Island, CA). The nucleotide sequences of the CD44 siRNA-1 were as follows: 5′-GAA CAA GGA GUC GUC AGA AAC UCC A-3′ (sense) and 5′-UGG AGU UUC UGA CGA CUC CUU GUU C-3′ (antisense). Transfection of cells with siRNAs was conducted using lipofectamine RNAiMAX (Invitrogen) in accordance with the manufacturer’s instructions. In brief, HUVECs at ~50% confluency were treated with a mixture of 25 nM siRNA and 1.25 μM Lipofectamine RNAiMAX in Opti-MEM (Invitrogen) for 3 h. The transfected cells were incubated for 2 days in a growth medium before cell adhesion assay and protein expression analysis.

CD44 plasmid construct and transfection. Total RNA purified from senescent HUVECs was used for the synthesis of first-strand cDNA using the Superscript III first-strand synthesis system for RT-PCR (Invitrogen). Full coding sequence of CD44 was PCR amplified from the first-strand cDNA using primers 5′-CCG GAT ATG GAC AAG TTT TGG TGG C-3′ and 5′-GCC GTC GGT GAG TAT GTC TTC-3′ (sense) and 5′-GTA GAG GCA GGG ATG ATG AGT-3′ (antisense). The PCR products were electrophoresed in a 1.0% agarose gel with a 100-bp Plus DNA ladder (Bioneer) as a size marker. The gel was ethidium bromide-stained, and the band intensities were determined using a Gel Doc system (Bio-Rad).

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for 30 min. Cells were then treated with 1 μg/ml plasmid DNA and 3 μg/ml NeoFectin in Opti-MEM for 4 h. The transfected cells were incubated for 2 days in a growth medium before cell adhesion assay and protein expression analysis.

**Immunohistochemistry.** Thoracic aortic tissues, isolated from Sprague Dawley rats of different ages, were kindly provided by Dr. Hae Young Chung, College of Pharmacy, Aging Research Resource Bank, Pusan National University (Busan, Korea). The aortic tissues were fixed in 4% paraformaldehyde in PBS and embedded with paraffin. Paraffin sections of 6 μm were subjected to immunohistochemistry. Tissue sections were blocked with 5% normal donkey serum (Jackson ImmunoResearch Laboratories) for 30 min at room temperature and then incubated overnight at 4°C with goat polyclonal antibody conjugated to horseradish peroxidase diluted 1:500 in immune IgG. The tissue sections were then incubated with donkey anti-goat antibody conjugated to horseradish peroxidase diluted 1:100. Controls were incubated with nonimmune IgG. The tissue sections were then incubated with donkey anti-goat antibody conjugated to horseradish peroxidase diluted 1:500 for 1 h at room temperature, followed by incubation for 5 min in 3,3′-diaminobenzidine solution (Vector Laboratories, Burlingame, CA). Sections were counterstained with mayer’s hematoxylin (Sigma-Aldrich). Tissue images were observed with an Eclipse 80i microscope (Nikon Instruments). The area of immunostaining in each tissue section was quantified using the LabWorks 4.5 software (Ultra-Violet Products, Upland, CA).

**Statistical analysis.** Data are presented as means ± SE of experiments performed in triplicate. Significant differences among the groups were determined using one-way ANOVA. Duncan’s multiple-range test was conducted if differences were identified between the groups at a significance level of P < 0.05.

### RESULTS

**Endothelial cell senescence promotes monocyte adhesion to endothelial cell surface.** Senescent HUVECs, prepared by continuous subculturing in vitro, showed typical characteristics of senescent cells, including increase in cell size and SA-β-gal activity (Fig. 1A). An experiment was conducted to compare

**Table 1. The “senescence-responsive cell adhesion genes” identified in this study**

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**Senescence-induced cell adhesion genes**

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YS, young cells under static conditions; YSS, YS, young cells under low shear stress (LSS) conditions; OS, senescent cells under static conditions; OSS, senescent cells under LSS conditions. Multiple gene probes were used for certain genes. Experimental conditions and gene selection method are described in Fig. 2.
the adhesion of monocytes (THP-1 cells) with young and senescent HUVECs that were or not stimulated with TNF-α. Monocytes prelabeled with a fluorescent dye were used for their selective detection on the nonfluorescent background of endothelial cells. After coincubation of endothelial cells and monocytes for 1 h, floating cells were washed off, and the fluorescent monocytes that adhered on the endothelial cell surface were observed under a fluorescence microscope. As shown in Fig. 1B, TNF-α significantly enhanced monocyte adhesion to both young and senescent endothelial cells. More interestingly, senescent endothelial cells showed a higher binding affinity for the monocytes compared with young cells, even in the absence of TNF-α stimulation (Fig. 1B). These results suggest that endothelial cell senescence in itself is an independent inducer of inflammatory leukocyte recruitment.

Identification of the senescence-induced cell adhesion genes. In an approach to identify the genes responsible for enhanced monocyte adhesion to senescent endothelial cells, the cDNA microarray data on the gene expression in young and senescent HUVECs under static and LSS conditions were analyzed (27). Cellular senescence significantly altered the expression levels of 2,351 genes under static conditions and 3,444 genes under LSS conditions. There were a total of 529 “senescence-responsive genes,” whose expression was commonly altered by cellular senescence under both static and LSS conditions. Among them, only the “cell adhesion genes” that are known to be functionally associated with cell adhesion were chosen based on the gene ontology annotation, as visualized with a Venn diagram (Fig. 2). Because the gene ontology information is still expanding, the selection of genes based on current annotation may not be perfectly correct. Possibly other genes ignored in this selection step may turn out to be important in the future. A total of 38 genes were selected as senescence-responsive cell adhesion genes by current ontology annotation and are listed in Table 1. They consisted of “senescence-induced” and “senescence-suppressed” cell adhesion genes. The senescence-induced cell adhesion genes included C1orf38, CD44, CEACAM1, COL4A6, DCBLD2, LPXN, NLGN1, and PVR.

Endothelial cell senescence upregulates CD44 gene expression. CD44 was chosen for further study as an example of senescence-induced cell adhesion genes, since the expression and function of this gene in endothelial cells are poorly understood, especially with respect to endothelial cell senescence in contrast to numerous studies that are reported with respect to other cell types (23, 29). To verify the cDNA microarray data on CD44 mRNA level, semiquantitative RT-PCR analysis was performed. Human CD44 pre-mRNA is known to undergo...
complex alternative splicing that results in various transcripts (Fig. 3A). Therefore, intron-spanning primers were designed so that most splicing variants could be identified based on the size of PCR amplicons (Fig. 3A). As shown in Fig. 3B, CD44 gene transcript 4 was determined to be the major variant expressed in endothelial cells. This variant is known to encode the standard isoform of CD44 protein (CD44s). The expression level of CD44 gene transcript 4 in senescent cells was much higher than that in young cells (Fig. 3B), whereas the effect of LSS on CD44 mRNA level was not significant (Fig. 3B). The mRNA level of GAPDH, a housekeeping gene, was not affected by cellular senescence or LSS (Fig. 3B). These results conform to the microarray data.

To analyze the protein levels of CD44, Western blotting was conducted. The CD44 antibody used in this experiment could potentially detect most isoforms of CD44 because it was generated against the NH2-terminal region, which is common to various isoforms. As shown in Fig. 3C, CD44 antibody detected a major protein band of ~90 kDa, which is bigger than the core molecular mass of CD44 protein (~37 kDa) whose predominant expression in endothelial cells was predicted from the mRNA analysis. Supporting the specificity of the antibody interaction, the ~90 kDa protein band was not detected when Western blot was conducted using the CD44 antibody preincubated with an excess of the immunogen peptide [Supplemental Fig. 1 (Supplemental data for this article may be found on the American Journal of Physiology: Heart and Circulatory Physiology website.)]. These results suggest that CD44 protein may be present in a highly glycosylated form in endothelial cells and migrates slowly on SDS-PAGE, as has been observed in other cells previously (8). The level of CD44 protein was significantly higher in the senescent cells than in young cells, irrespectively of LSS treatments (Fig. 3C) and was in accordance with mRNA levels (Fig. 3B). A high-level expression of CD44 in senescent endothelial cells was also verified by immunocytochemistry (Fig. 4).

CD44 mediates monocytes adhesion to endothelial cells. To examine if CD44 is responsible for the enhanced monocyte binding to senescent endothelial cells, two different approaches were followed. First, endothelial cells were pretreated with CD44 antibody before the cell adhesion assay. Because CD44 protein is anchored in the plasma membrane through the transmembrane domain at the COOH-terminus while the NH2-terminal region is available for binding to other cells or to extracellular matrix, preoccupancy of the NH2-terminal region of CD44 by the antibody can prevent adhesion of monocytes to endothelial cells. In accordance with this notion, both basal and TNF-α-stimulated monocyte adhesions to senescent endothelial cells were significantly reduced by CD44 antibody and not by nonimmune IgG (Fig. 5, A and B). In the second approach, cells were treated with siRNA to downregulate the gene expression of CD44 specifically. CD44 siRNA with the nucleotide sequence in Exon 1 of human CD44 gene was used. As shown in Fig. 5, A and C, pretreatment of senescent endothelial cells with CD44 siRNA-1 but not a negative control oligonucleotide duplex significantly prevented the monocyte adhesion in the absence and presence of TNF-α. Selective gene silencing effect of CD44 siRNA was confirmed at the mRNA and protein levels (Fig. 5, D and E). Experiments using two more CD44 siRNAs with different nucleotide sequences provided similar results (Supplemental Fig. 2).

![Fig. 4. Immunocytochemical detection of CD44 in cultured HUVECs. Note that green fluorescence signal detected by CD44 antibody was much stronger in senescent cells than in young cells. The fluorescence signal was almost absent when nonimmune IgG was used in place of CD44 antibody (insets), supporting the specificity of this immunocytochemical method.](http://www.ajpheart.org/)

*Fig. 4. Immunocytochemical detection of CD44 in cultured HUVECs. Note that green fluorescence signal detected by CD44 antibody was much stronger in senescent cells than in young cells. The fluorescence signal was almost absent when nonimmune IgG was used in place of CD44 antibody (insets), supporting the specificity of this immunocytochemical method.*
A critical role of CD44 in monocyte adhesion was further examined in young endothelial cells transfected with the CD44 plasmid construct. Transfection of young HUVECs with the CD44 construct increased CD44 expression and led to a significant enhancement of monocyte adhesion, even in the absence of TNF-α/H9251 stimulation (Fig. 6A). These results were reproduced in HAECs that are thought to be more relevant to the pathological conditions of atherosclerosis (Fig. 6B).

Endothelial CD44 expression increases with age in rats. The physiological relevance of the above findings was further sought with animal models. The aortic tissues isolated from the rats of different age were subjected to immunohistochemistry and Western blotting to examine the protein expression of CD44. As shown in Fig. 7A, endothelial CD44 was clearly detected in the aortic tissue sections of old animals but not in those of young animals. Semiquantitative immunocytochemistry revealed an age-dependent increase of CD44 protein level in the aortic endothelium (Fig. 7B). In addition, similar results were obtained by quantitative Western blotting of aortic tissues (Fig. 7C).

DISCUSSION

Keeping other predisposing factors equal, the incidence of atherosclerosis is known to increase with age. Organism aging is accompanied by a significant accumulation of senescent cells in tissues that show altered phenotypes distinct from those of young healthy cells (3, 14, 33). Thus cellular senescence has been proposed as an important contributor for the development of age-associated diseases, including atherosclerosis. The present study demonstrated that endothelial cell senescence in itself promotes binding of inflammatory monocytes, recognized as the critical initiation step of atherosclerosis, by way of inducing expression of “cell adhesion genes,” irrespective of LSS treatments.

The adherence of mononuclear leukocytes to the endothelial cell surface is mediated by multiple adhesion molecules whose expression is induced by inflammatory stimuli. Especially vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin are known to be induced by cytokines and play a critical role in leukocyte migration in subendothelial sites of blood vessels (28). Of
interest, the oscillatory shear stress had been shown to stimulate the adherence of mononuclear leucocytes to the endothelial surface by inducing expression of those adhesion molecules (5, 18). However, the results from the current study showed that the expression of VCAM-1, ICAM-1, and E-selectin was insensitive to the replicative senescence of endothelial cells (data not shown). Instead, this study identified a group of senescence-induced cell adhesion genes that included CD44 (Table 1).

Endothelial CD44 is known to play a role in cell proliferation and locomotion that are required for such processes as blood vessel wall morphogenesis (15), angiogenesis (4), and tumor metastasis (17). Previous studies have also shown that there is a significant reduction of atherosclerotic lesion formation in CD44-null mice compared with CD44 heterozygote and wild-type littermates (7). In addition, the expression levels of CD44 have been found to be significantly higher in diseased arterial tissues than in normal tissues (20), implicating an association of CD44 expression levels with the development of atherosclerosis.

The results from the current study further showed that endothelial CD44 is highly expressed in senescent endothelial cells (Figs. 3 and 4), and it can mediate the monocyte adhesion (Figs. 5 and 6). An age-dependent increase of endothelial CD44 expression was also shown in animal models (Fig. 7). Thus it is suggested that endothelial CD44, which is highly expressed in old animals, because of cellular senescence, may contribute to the progression of atherosclerosis by recruiting activated leukocytes to endothelium.

Hyaluronan, an important glycosaminoglycan constituent of extracellular matrix, mediates both homotypic and heterotypic cell-cell aggregations by providing a cross-bridge between cells (1). CD44 serves as the principal receptor for hyaluronan (11). Therefore, the hyaluronan-mediated cell-cell aggregations may require the expression of CD44 in both cell types. In this regard, CD44, highly expressed in endothelial cells in response to inflammatory cytokines or as a result of endothelial cell senescence, may directly enhance the recruitment of activated leukocytes. Alternatively, endothelial CD44-hyaluronan interactions may activate intracellular signals leading to the stimulation of processes relevant to cell-cell interactions (29).

Until the current study, enhanced monocyte adhesion to senescent endothelial cells had been attributed to the impairment of nitric oxide (NO) production (24). In fact, previous studies have demonstrated that LSS prevents monocyte adhesion to endothelial cells through a mechanism involving NO production (34). Then, is CD44 expression inhibited by LSS or NO? The results of the present study, however, indicated that this would not be the case, because the CD44 expression level was not affected by LSS (Fig. 3), which increased NO production even in senescent endothelial cells (27). Thus the inhibitory effect of LSS and/or NO on leukocyte adhesion, if any, may not be related to CD44 suppression. Therefore, intervention of CD44 expression or its function would provide a novel and independent strategy for the prevention of monocyte adhesion to senescent endothelial cells, thus preventing the initiation of the atherosclerotic process.

In conclusion, the present study identified senescence-induced cell adhesion genes potentially responsible for the proadhesive phenotypes of senescent endothelial cells.
expression and function of CD44 was investigated in detail as an example of such genes. The results indicated a critical role of CD44 protein in leukocyte adhesion and highlighted the remarkable increase of endothelial CD44 during cellular senescence and animal aging. Although this study focused on CD44, many other senescence-induced cell adhesion genes may potentially contribute to the proadhesiveness of senescent endothelial cells, thus necessitating a need for further studies to examine this possibility. The senescence-induced cell adhesion genes identified in this study may provide novel targets for the prevention of atherosclerosis.

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DISCLOSURES
No conflicts of interest are declared by the authors.

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