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Senescent endothelial cells are prone to TNF-α-induced cell death due to expression of FAS receptor

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ABSTRACT

The senescent endothelial cells show various phenotypes which can increase the incidence of inflammatory cardiovascular diseases, but the fundamental basis for such phenotypic changes of senescing cells remains to be elucidated. This study was undertaken to find transmembrane receptors that might be highly expressed in senescent endothelial cells and play a key role in cell death signal transduction. Comparison of mRNA expression in young and senescent human umbilical vein endothelial cells, using a cDNA microarray method, provided a list of transmembrane receptors including the FAS receptor (tumor necrosis factor receptor superfamily member 6) whose expression levels were significantly increased by cellular senescence. Additional studies focused on FAS demonstrated that a high expression of FAS receptor in senescent endothelial cells is responsible for the susceptibility to apoptotic cell death, as the siRNA-mediated suppression of FAS expression in senescent cells prevented the cell death, and overexpression of exogenous FAS in young cells increased cell death. We also verified that FAS expression level was closely associated with the activation of caspase-3 and caspase-9 involved in apoptosis. The senescence-induced transmembrane receptors including the FAS receptor may provide novel therapeutic targets to prevent cardiovascular diseases.

1. Introduction

Aging is a major risk factor for the development of atherosclerosis [1] and increasing attention has been given to endothelial senescence as a fundamental mechanism that can explain the increasing incidence of cardiovascular diseases with age [2]. Indeed, senescent endothelial cells are readily detectable at atherosclerotic lesions in the vessels of elderly people [3–5]. Like other somatic cells, after a certain number of cell divisions, endothelial cells also enter a state of replicative senescence characterized by morphological changes and functional degeneration [6]. In addition, premature cellular senescence can be accelerated by various stress factors [7,8]. General features of senescent cells include increases in cell size, protein content per cell and senescent-associated β-galactosidase activity, and the slowdown of cell division [9].

The capability of endothelial cells to produce NO is weakened as a consequence of cellular senescence and this phenomenon has been attributed to the lower expression of nitric oxide synthase 3 (NOS3) in senescent endothelial cells compared to young cells [10]. Our studies have shown that additional important reason is the decreased expression of argininosuccinate synthetase 1 [9,11] involved in providing L-arginine, the endogenous substrate of NOS3 [12]. In fact, inflammatory monocyte adhesion to the endothelial cell monolayer was greatly affected by the expression levels of argininosuccinate synthetase 1 as well as NOS3 [13]. It has also been demonstrated that the adhesion of inflammatory monocytes to endothelial cells progressively increases with the senescing of endothelial cells [14]. We explained this phenomenon based on the increases of cell adhesion molecules such as CD44 in senescent endothelial cells compared to younger cells [15]. Of course, the increased monocytic adhesion to senescent endothelial cells might be partly attributed to decreased NO production because there was a negative correlation between NO production and monocyte adhesion [13]. The senescence of endothelial cells is also accompanied by an increased susceptibility to apoptotic cell death,

Abbreviations: EGFP, enhanced green fluorescent protein; FAS, tumor necrosis factor receptor superfamily member 6; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HUVECs, human umbilical vein endothelial cells; LSS, laminar shear stress; NO, nitric oxide; NOS3, nitric oxide synthase 3; RT-PCR, reverse transcriptase-polymerase chain reaction; siRNA, small interfering RNA; TNF, tumor necrosis factor.

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and this phenomenon has been attributed previously to insufficiency of NO production [10], and the deteriorated mitochondrial redox state [16].

Although these previous studies including ours have presented molecular mechanisms involved in the pro-atherogenic effects of endothelial cell senescence, an important question still remains as to the transmembrane receptors that might lead such phenotypic changes. This issue was addressed in the present study by using young and senescent human umbilical vein endothelial cells (HUVECs) cultured *in vitro*. The cDNA microarray analysis identified a list of transmembrane receptors including the FAS receptor (tumor necrosis factor receptor superfamily member 6) whose expression levels were significantly upregulated in senescent endothelial cells. Additional studies focused on FAS suggested that that FAS receptor highly expressed in senescent endothelial cells may contribute to the susceptibility to apoptotic cell death.

2. Materials and methods

2.1. Cell culture, laminar LSS treatments, and gene expression profiling

HUVECs obtained from Clonetics Cambrex (Rockland, ME, USA) were cultured in EBM-2 medium containing endothelial growth supplements (Clonetics Cambrex), 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) and antibiotics (100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 0.25 μg mL^{-1} amphotericin B) on 0.2% gelatin-coated culture dishes at 37 °C and 5% CO_2 [17]. Cells were repeatedly subcultured at ratios of 1 to 3–5 in the same media until they exhibited senescence-associated phenotypes [9]. The population doubling level (PDL) was calculated using the following formula: PDL = log [the final cell count/the starting cell count]/log 2. After changing the growth medium with a fresh one, young (PDL 8–12) or senescent HUVECs (PDL 35–45) on a 100 mm-culture dish (BD Biosciences, San Jose, CA, USA) were exposed to steady LSS at 12 dyn cm^{-2} or kept under static conditions for 24 h. LSS was provided by rotating a Teflon cone (0.5° cone angle) mounted onto a culture dish as previously described [18,19]. Total cellular RNA was extracted from cells using the RNeasy kit (Qiagen, Valencia, CA, USA) and gene expression profiles were analyzed on a GeneChip® HG-U133 Plus 2.0 (Affymetrix, Santa Clara, CA, USA) [9]. The complete datasets were deposited in the Gene Expression Omnibus database [Accession Number, GSE13712]. Each of the three experimental samples was compared with each of the three control samples resulting in nine pair-wise comparisons. Expression of a gene was considered to be significantly altered if the p value for the average fold changes were <0.05.

2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was conducted using the GeneAmp® PCR system 9700 (Applied Biosystems, Foster city, CA, USA) in a reaction mixture (20 μL) containing *Maxime* RT-PCR PreMix (iNtRON 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 FAS gene (GeneBank accession number, variant 1, NM000043.3; variant 2, NM152871.1; variant 3, NM152872.1; 5′-CTT TCA CTT CGG AGG ATT GC-3′ (sense) and 5′-AGC CAT GTC CTT CAT CAC AC-3′ (antisense); GAPDH (GeneBank accession number, NM002046) 5′-GCC AAA AGG GTC ATC ATC TC-3′ (sense) and 5′-GTA GAG GCA GGG ATG ATG TTC-3′ (antisense). The PCR products were electrophoresed on a 1.0% agarose gel with a 100 bp Plus DNA ladder (Bioneer) as a size marker. The gel was stained with ethidium bromide and the band intensities were determined using a Gel Doc system (Bio-Rad, Hercules, CA, USA).

2.3. Western blotting

Western blotting of cell lysates was done as previously described [20]. Cells were lysed in a lysis buffer (10 mM Tris–Cl, 150 mM NaCl, 5 mM EDTA, 0.1% Sodium dodecyl sulfate, 1% TritonX-100, 1% deoxycholate pH 7.2) supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Roche, Mannheim, Germany). 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, USA) for 1 h at room temperature. The immunoreactive bands were detected using a picoEPD Western Reagent kit (ELPIS-Biotech, Daejeon, Korea) and analyzed using the NIH Image program. Mouse monoclonal antibody generated against the C-terminal region of human FAS was purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). And the monoclonal β-actin antibody was from Sigma–Aldrich (St. Louis, MO, USA).

2.4. Cell viability determination

Cell viability was assayed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). Briefly, cells were washed with PBS buffer twice and MTT solution at a final concentration of 1 mg mL^{-1} in fresh growth medium was added to each well. After the cells were incubated for 3 h at 37 °C in a 5% CO_2 incubator, the supernatants were removed and isopropanol was added and mixed thoroughly using a pipette to dissolve the purple formazan crystals. The optical density (OD) of the plate was measured at 595 nm by a BioRad Model 680 microplate reader (Bio-Rad). Cell viability (%) = [(OD sample − OD blank)/OD control] × 100%.

2.5. Transfection with a small interfering RNA (siRNA)

FAS siRNA (#1299001, HSS100599) with nucleotide sequences corresponding to the coding region of a human FAS gene transcript (NM000043.3, NM152871.1, NM152872.1) and a negative control siRNA (#12935200) with scrambled sequences were purchased from Invitrogen (Grand Island, CA, USA). The nucleotide sequences of the FAS siRNA were as follows: 5′-GGG AUU GGA AUU GAG GAA GAC UGU U-3′ (sense) and 5′-AAC AGU CUU CCU CAA UUC CAA UCC C-3′ (antisense). Transfection of HUVECs with siRNAs was performed using lipofectamine RNAiMAX (Invitrogen). Briefly, cells at ~50% confluency on an 12-well cell culture plate (SPL, Pocheon, Korea) were treated with a mixture of 100 nM siRNA and 1.25 μL mL^{-1} Lipofectamine RNAiMAX in 500 μL of Opti-MEM (Invitrogen) for 4 h, followed by incubation in a growth medium for 48 h.

2.6. Transfection with a plasmid encoding FAS

Plasmid clones of human FAS in pEZ-M02 vector (#EX-G0198-M02) and EGFP in pReceiver-M02 vector (#EX-EGFP-M02) were purchased from GeneCopoeia (Rockville, MD, USA). The plasmids were propagated in the *Escherichia coli* strain DH5α (TaKaRa Bio Inc., Shiga, Japan) and purified using the Maxi-Prep DNA purification kit (Qiagen). The FAS coding sequence was verified to be identical to the reference sequence (NM000043). Transfection of HUVECs with the FAS expression plasmid or empty control vector was performed using TrueFect™ (United BioSystems Inc., Rockville, MD, USA). Briefly, cultured cells at ~90% confluency were treated with 1 μg mL^{-1} plasmid DNA and 3 μL mL^{-1} TrueFect™ in Opti-MEM for 4 h.

2.7. Statistical analysis

Data are presented as the Means ± SEM 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 $p < 0.05$.

3. Results and discussion

When young HUVECs are continuously subcultured *in vitro*, they show typical signs of senescent cells including an increase of cell size, increase of the senescence-associated-β-galactosidase activity and a slowdown of the cell division rate [15]. The senescence of endothelial cells is also accompanied by a change in phenotype characterized by the decreased ability to produce nitric oxide (NO) [9,10], increased adhesion potential to recruit inflammatory leukocytes [14], susceptibility to apoptotic cell death [10], and so on.

In an approach to identify the genes responsible for such phenotypic changes of senescing endothelial cells, we analyzed cDNA microarray data from the gene expression of young and senescent HUVECs under static and LSS conditions [9]. Replicative senescence of endothelial cells altered the expression levels of 2351 genes under static conditions and 3444 genes under LSS conditions. The number of genes whose expression was commonly altered by cellular senescence under both static and LSS conditions was 543 and these genes were considered as 'senescence-induced genes'. These genes include 16 transmembrane receptors shown in Table 1. Cellular senescence was found to increase the expression levels of FAS, IL1RL1, NPC1 and NPG1. In contrast, the expression levels of ACVR2A, CD40, CELSR1, EFEMP2, EPHB1, FBN1, IL1R1, INSR, ITPR2 and LYVE1 were significantly suppressed by endothelial cell senescence. Altered expressions of these transmembrane receptors may be associated with the phenotypic changes of endothelial cells due to senescence.

The FAS receptor was chosen for further study as an example of 'senescence-induced transmembrane receptors' because the fold-change of its expression level was very large (Table 1). In order to verify the cDNA microarray data for the FAS mRNA level, semi-quantitative RT-PCR analysis was performed. As shown in Fig. 1A, the expression level of FAS gene transcripts in senescent cells was much higher than that in young cells. Although FAS expression has been previously reported to be sensitively attenuated by LSS [21], we could not observe such a phenomenon. The mRNA level of GAPDH, a housekeeping gene, was unaffected by cellular senescence or LSS. These results conform to the microarray data. The protein level of FAS was additionally analyzed by western blot. The FAS antibody generated against the N-terminal region was used in this experiment. As shown in Fig. 1B, the protein level of FAS was significantly higher in the senescent cells than in the young cells, regardless of the LSS treatments, in agreement with the mRNA levels observed above. The protein level of β-actin, a loading control, was not altered by cellular senescence or LSS.

Endothelial cells are known to be relatively resistant to apoptotic cell death but they commit apoptosis to a substantial degree when exposed to serum depletion [22], high glucose [23], and oxidized low-density lipoprotein [24]. Evidence has supported that oxidative stress and redox signaling pathways are involved in endothelial apoptosis [25,26]. Endothelial apoptotic cell death is known to be increased by replicative senescence [10,16].

To compare the susceptibility of young and senescent HUVECs to apoptotic cell death, these cell were treated with TNF-α at 1–100 ng mL^{-1} for 24 h. As shown in Fig. 2A, the young cells were very insensitive to TNF-α treatment and their viability was as high as the control cells treated with vehicle only. In contrast, the viability of the senescent cells was significantly lowered by TNF-α treatment at 10–100 ng mL^{-1}. These data conform to previous reports that showed increased apoptosis due to replicative senescence of endothelial cells [10,16].

FAS receptor is a death receptor on the surface of cells that initiates programmed cell death (apoptosis) when bound to its ligand FAS-L. Previous studies have reported that FAS has a critical role in endothelial cell apoptosis induced by oxidized low density lipoprotein [24]. Therefore, we hypothesized that enhanced expression of FAS may sensitize senescent endothelial cells to apoptotic stimuli.

To examine if FAS is responsible for the enhanced apoptotic cell death of senescent endothelial cells, senescent HUVECs were treated with siRNA to downregulate the gene expression of FAS specifically. FAS siRNA (siFAS) with the nucleotide sequence of Exon 2 in the FAS gene was used. As shown in Fig. 2B, transfection of senescent endothelial cells with siFAS resulted in the almost complete

Table 1
The 'senescence- responsive transmembrane receptors' identified in this study.

Probe ID	Relative Expression Level				Gene Symbol	Gene Title
	Young		Senescent			
	Static	LSS	Static	LSS		
Senescence-induced transmembrane receptors						
215719_x_at	1.00	1.52	3.05	5.26	FAS	Fas (TNF receptor superfamily, member 6)
216252_x_at	1.00	1.76	3.02	5.98	FAS	Fas (TNF receptor superfamily, member 6)
234066_at	1.00	1.76	4.65	5.40	IL1RL1	Interleukin 1 receptor-like 1
242809_at	1.00	1.76	4.25	4.82	IL1RL1	Interleukin 1 receptor-like 1
202679_at	1.00	2.79	1.75	5.43	NPC1	Niemann-Pick disease, type C1
206237_s_at	1.00	0.55	2.44	1.95	NRG1	neuregulin 1
Senescence-suppressed transmembrane receptors						
228416_at	1.00	1.20	0.73	0.81	ACVR2A	activin A receptor, type IIA
35150_at	1.00	1.10	0.47	0.43	CD40	CD40 molecule, TNF receptor superfamily member 5
41660_at	1.00	2.03	0.04	0.38	CELSR1	cadherin, EGF LAG seven-pass G-type receptor 1 (flamingo homolog, Drosophila)
209356_x_at	1.00	0.89	0.61	0.54	EFEMP2	EGF-containing fibulin-like extracellular matrix protein 2
210753_s_at	1.00	0.71	0.35	0.07	EPHB1	EPH receptor B1
235318_at	1.00	1.90	0.40	0.62	FBN1	fibrillin 1
202948_at	1.00	2.28	0.37	0.39	IL1R1	interleukin 1 receptor, type I
226450_at	1.00	1.98	0.68	0.77	INSR	insulin receptor
202661_at	1.00	1.29	0.69	0.67	ITPR2	inositol 1,4,5-triphosphate receptor, type 2
219059_s_at	1.00	1.51	0.21	0.27	LYVE1	lymphatic vessel endothelial hyaluronan receptor 1

Multiple gene probes were used for some genes.

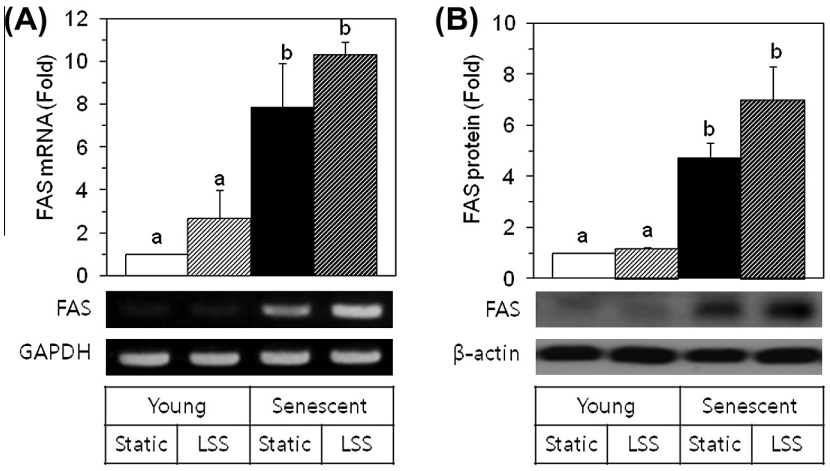

Fig. 1. Effects of replicative senescence and LSS on the expression of FAS in HUVECs. Young and senescent HUVECs were subjected to LSS at 12 dyn cm^{-2} or maintained under static conditions for 24 h. The expressions of FAS, GAPDH and/or β-actin at the mRNA and protein levels were monitored by RT-PCR (A) and Western blotting (B), respectively. Data are presented as Mean ± SE ($n = 3$). Data not sharing the same letter differ significantly from each other ($p < 0.05$).

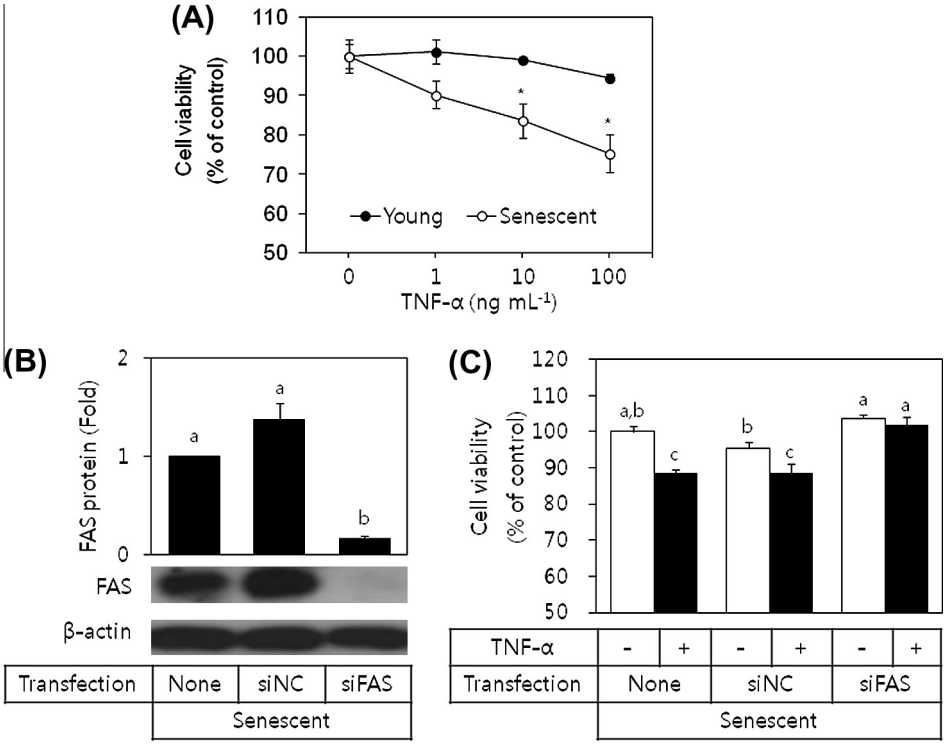

Fig. 2. The siRNA-mediated knockdown of FAS prevents the death of senescent HUVECs stimulated by TNF-α. In panel A, young and senescent HUVECs were treated with 1–100 ng mL^{-1} TNF-α or vehicle for 24 h. In panels B and C, senescent cells were transfected with a siRNA for FAS (siFAS) or negative control siRNA (siNC), incubated for 48 h, and then treated with TNF-α at 100 ng mL^{-1} for additional 24 h. Protein expression was analyzed by Western blot (B), and cell viability was determined by MTT assay (A, C). Data are presented as Mean ± SE ($n = 3$). Data not sharing the same letter are significantly different from each other ($p < 0.05$).

depletion of FAS protein 48 h later compared to the no transfection or negative control siRNA (siNC) transfection. The β-actin protein level was not altered by siFAS or siNC transfection. These results show the selectivity and efficiency of the siFAS used in this study. The senescent endothelial cells with different FAS expression levels were then treated with 100 ng mL^{-1} TNF-α for an additional 24 h to induce apoptotic cell death. As shown in Fig. 2C, TNF-α treatment decreased the viability of the no transfection cells and siNC-transfected cells significantly. However, this phenomenon was not observed in cells transfected with siFAS, indicating FAS has a key role in TNF-α-induced apoptotic death of senescent endothelial cells.

The critical role of FAS in endothelial cell death induced by TNF-α was further examined in young HUVECs transfected with the plasmid construct encoding FAS. The cells transfected with the EGFP construct provided controls, together with the no transfection cells. The transfection efficiency was estimated to be 30–40% by counting EGFP expressing cells (Fig. 3A). Transfection of young HUVECs with the FAS construct, but not the EGFP construct, increased FAS protein expression markedly as expected (Fig. 3B).

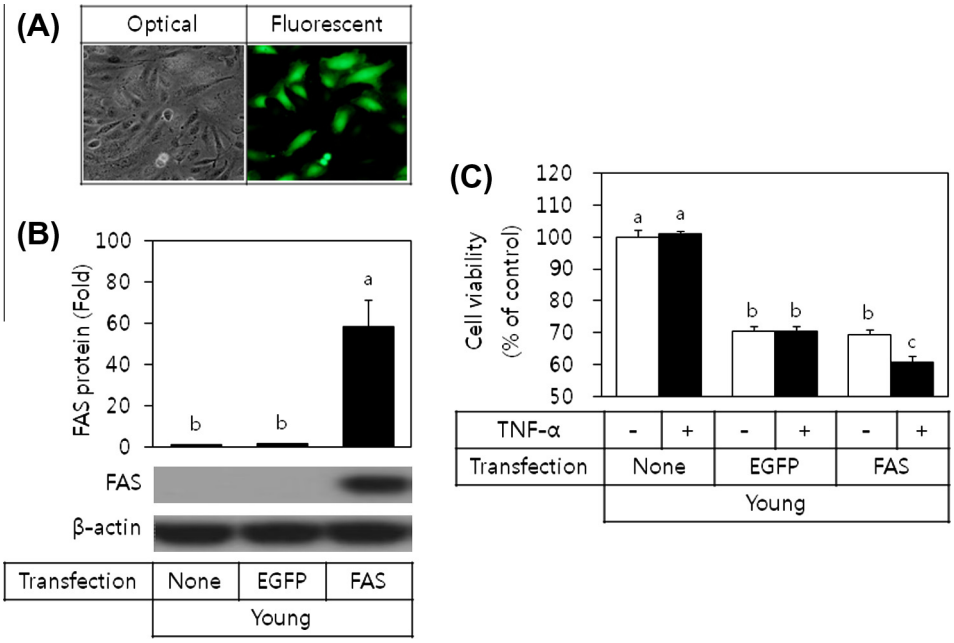

Fig. 3. Overexpression of exogenous FAS in young endothelial cells increases cell death in response to TNF-α. Young HUVECs were transfected with the plasmid encoding FAS or EGFP and incubated in growth medium for 48 h and then stimulated with TNF-α at 100 ng mL^{-1} for additional 24 h. Optical and fluorescent images of the cells transfected with the EGFP-encoding plasmid are shown (A). Protein expression was analyzed by western blot (B), and cell viability was determined by MTT assay (C). Data are presented as Mean ± SE ($n = 3$). Data not sharing the same letter are significantly different from each other ($p < 0.05$).

The young cells with different FAS expression levels were then treated with 100 ng mL^{-1} TNF-α for additional 24 h to induce apoptotic cell death (Fig. 3C). The transfection procedure itself appeared to decrease cell viability significantly; however, the effect of TNF-α inducing cell death was clearly observed only in cells overexpressing FAS (Fig. 3C).

Tumor necrosis factor (TNF) super family members regulate cell proliferation, survival and death [27]. Death signaling is initiated when ligands such as TNF-α, FAS-L and TRAIL bind to their receptors (TNFR1, FAS, or DR4 and DR5). Although the FAS expression level was critical in cell death stimulated by TNF-α, this effect may be indirect as in the case of oxidized low-density lipoprotein [24], because the specific ligand of FAS is FAS-L. Nonetheless, the current study demonstrated that a high expression of FAS receptor in senescent endothelial cells is a key contributor to increased susceptibility to apoptotic cell death. Supporting this notion, the siRNA-mediated suppression of FAS expression in senescent HUVECs prevented the cell death triggered by TNF-α (Fig. 2), and overexpression of exogenous FAS in young cells increased cell death induced by TNF-α (Fig. 3). We also verified that FAS expression level was closely associated with the activation of caspase-3 and caspase-9 involved in apoptosis (Supplementary Fig. S1).

Although this study focused on FAS only, many other 'senescence-induced transmembrane receptors' such as IL1RL1, NPC1 and NRG1 may also contribute to the phenotypic changes of endothelial cells. IL1RL1 (Interleukin 1 receptor-like 1), also known as ST2, is a member of the Toll-like receptor superfamily. Its ligand is the cytokine Interleukin-33. Binding of Interleukin-33 to the IL1RL1 receptor has been shown to provide cardioprotective effects [28]. NPC1 (Niemann–Pick disease, type C1) was identified because its mutation results in Niemann–Pick disease, type C. Niemann–Pick disease, type C is a rare neurovisceral lipid storage disorder characterized by the accumulation of lipid products in late endosomes and lysosomes. NPC1 is a integral membrane protein containing sequence motifs consistent with a role in intracellular transport of cholesterol to postlysosomal destinations [29]. NRG1 (Neuregulin 1) is a protein in the neuregulin family that acts on the EGFR family of receptors. Neuregulin 1 is essential for the normal development of the nervous system and heart [30]. Because IL1R1, NPC1 and NRG1 were observed to be expressionally upregulated by endothelial senescence, their functional roles related to vascular aging would be an interesting subject for future studies.

In conclusion, the present study identified 'senescence-induced transmembrane receptors' potentially responsible for the proatherogenic phenotypes of senescent endothelial cells. This study further showed that the high expression of FAS in senescent endothelial cells is associated with the susceptibility to apoptotic stimuli such as TNF-α. These senescent-induced transmembrane receptors may provide novel therapeutic targets to prevent cardiovascular diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.052.

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