Age-Related Decline in Reendothelialization Capacity of Human Endothelial Progenitor Cells Is Restored by Shear Stress

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Abstract—Aging is associated with dysfunction of endothelial progenitor cells (EPCs), and shear stress has a beneficial impact on EPC function; however, the effects of aging and shear stress on the endothelial repair capacity of EPCs after arterial injury have not been reported. Here we investigated the influence of aging and shear stress on the reendothelialization capacity of human EPCs and the related molecular mechanism. Compared with EPCs isolated from young subjects, EPCs from the elderly displayed an impaired migration and adhesion in vitro and demonstrated a significantly reduced reendothelialization capacity in vivo after transplantation into nude mice with carotid artery denudation injury. Shear stress pretreatment enhances the migration, adhesion, and reendothelialization capacity in both young and elderly EPCs; however, it was to a greater extent in EPCs from the elderly. Although basal CXC chemokine receptor 4 (CXCR4) expression was similar in EPCs from the 2 age groups, the stromal cell derived factor 1-induced CXCR4 and Janus kinase 2 phosphorylations were much lower in the elderly than in young EPCs. Shear stress treatment upregulated CXCR4 expression and phosphorylation and, importantly, restored the stromal cell-derived factor 1/CXCR4-dependent Janus kinase 2 phosphorylation in the elderly EPCs. Furthermore, short hairpin RNA-mediated knockdown of CXCR4 expression or pretreatment with Janus kinase 2 inhibitor diminished the enhancement in the migration, adhesion, and reendothelialization capacity of the elderly EPCs from shear stress treatments. Thus, our study demonstrates that upregulation of the CXCR4/Janus kinase 2 pathway by shear stress contributes to the enhanced reendothelialization capacity of EPCs from elderly men. (***Hypertension***. **2012;59:1225-1231.)** ● **Online Data Supplement**

Key Words: aging ■ endothelial progenitor cells ■ CXCR4 ■ shear stress ■ endothelial repair

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Aging is a well-recognized risk factor for cardiovascular disease.[1,2] The impact of aging, a traditional detrimental factor, for the increased development of cardiovascular disease is initiated by abnormalities in structure and function of the vascular endothelium.[3–5] Thus, it is of particular importance to maintain the integrity of the vascular endothelium after arterial injury with aging.

Accelerated reendothelialization is an important therapeutic means for repair of injured artery. Accumulating evidence indicates that circulating endothelial progenitor cells (EPCs) provide an endogenous repair mechanism to counteract ongoing risk factor–induced endothelial injury and to replace dysfunctional endothelium,[6–10] thus suggesting an important role of circulating EPCs for restoration of the integrity of the vascular endothelium with aging. Previous studies showed that aging leads to a reduction in the number of circulating EPCs, and aging is associated with dysfunctional EPCs in both healthy persons and patients with cardiovascular disease,[11–16] which is, at least in part, responsible for the development of age-related endothelial injury in humans.[17–19] However, the mechanism underlying age-related EPC dysfunction is not fully understood. It is, therefore, essential to search for a novel approach to improve the functional potential of EPCs in elderly individuals, with the aim of enhancing EPC-based endothelial repair and reducing the occurrence of cardiovascular disease.

The pharmacological therapy and the gene transfer intervention are the most popular methods to enhance the EPC function for their therapeutic potentials. However, less is focused on the nonpharmacologic interventions to regulate

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the function of EPCs. It is generally accepted that shear stress as a nonpharmacologic intervention measure contributes to the maintenance of homeostasis in the vascular endothelium.[20] Accumulating evidence indicates that the beneficial effect of shear stress on the vascular endothelium is, at least in part, related to shear stress-mediated upregulation of EPC function.[21–25] However, the molecular mechanisms underlying the favorable effect of shear stress on EPCs need to be further investigated.

CXC chemokine receptor 4 (CXCR4) is a 7-transmembrane G protein–coupled receptor, and the ligand for CXCR4 is chemokine stromal cell derived factor 1 (SDF-1). There is increasing evidence to show that CXCR4 is a key regulator of homing and retention of EPCs at the sites of injured artery,[26–28] suggesting that an impaired CXCR4 signaling may lead to a decrease in endothelial repair capacity of EPCs. Our recent study indicated that impairment of CXCR4-mediated Janus kinase 2 (JAK-2) phosphorylation is involved in the aged-related reduction in endothelialization capacity of EPCs[29]; however, the role of CXCR4 phosphorylation in the regulation of CXCR4/JAK-2 signaling is unknown. It is also not clear whether shear stress could enhance reendothelialization capacity of EPCs derived from the elderly. Based on the previous observations, we hypothesized that the impaired reendothelialization capacity of EPCs derived from elderly persons is related to the decreased CXCR4 phosphorylation, and shear stress can be used to promote reendothelialization capacity of EPCs from elderly persons, which correlates to increasing CXCR4 signaling. To test these hypotheses, in vivo reendothelialization capacity and CXCR4 signaling of EPCs were examined in both elderly and young subjects. Then, the EPCs from the elderly were exposed to an environment of shear stress produced by the biomimic device. Under physiological conditions of shear stress, the human EPCs were tested in vitro for their ability to affect the CXCR4 signaling, as well as migration and adhesion function. In addition, we also evaluated the effect of transplantation of shear stress-treated EPCs from elderly persons on in vivo reendothelialization capacity in a nude mouse model of carotid injury. The present study may add valuable information to our understanding of age-related endothelial injury and provide a novel therapeutic strategy to counteract the decline in EPC function in humans with aging.

Materials and Methods

Subjects Characteristics

Elderly (age, 68.4±2.5 years; n=10) and young (age, 27.3±3.0 years; n=10) healthy male subjects without clinical evidence of cardiovascular risk factors and significant medical history were enrolled into the study. Peripheral venous blood samples were obtained for EPC isolation. The study protocol was approved by the ethics committee of our hospital, and written informed consent was obtained from all of the study subjects. Detailed methods for the present study are provided in the online-only Data Supplement Materials and Methods section.

Statistical Analysis

Statistical analyses were performed using SPSS 17.0 software (SPSS Inc). All of the data are reported as mean (SD) unless stated otherwise. Statistical significance was evaluated by means of a Student *t* test or ANOVA. A value of $P<0.05$ was considered statistically significant.

Results

Study Subjects

The baseline characteristics were within normal range and not different between elderly and young subjects except with regard to age (Table S1).

In Vitro Functions and In Vivo Reendothelialization Capacity of EPCs From Elderly Human Subjects Are Impaired

The percentage of $CD34^+KDR^+$ cells was significantly lower in the peripheral blood mononuclear cells from the elderly ($P<0.05$; Figures S1A and S2). We then grew EPCs by culturing peripheral blood mononuclear cells in specific medium for 7 days. Cultured EPCs were defined as cells dually positive for low-density lipoprotein from human plasma, acetylated, DiI complex uptake and fluorescein isothiocyanate–labeled BS-1 lectin binding or by flow cytometry analysis (Figure S3). In a modified Boyden chamber assay, the basal level of migration was lower ($P<0.05$), and the fold change of SDF-1–induced increase of migration was lesser (0.33±0.11 versus 0.78±0.21; $P<0.01$) in EPCs from the elderly than those from young subjects (Figure S1B). The EPC adhesion activity was evaluated by applying DiI-labeled EPCs onto tumor necrosis factor-α–prestimulated monolayer human umbilical vein endothelial cells, subsequently counting the adherent cells under the fluorescent microscope; the numbers of adherent EPCs from elderly were fewer than those from young subjects ($P<0.01$; Figure S1C). Consistently, the migration and adhesion activities of late EPCs from the elderly were significantly lower than those from young subjects (Figure S4). Collectively, these results suggest that aging is associated with a reduced number of circulating EPCs and impaired function of cultured EPCs.

Transplantation of EPCs from young people but not EPCs from the elderly markedly accelerated reendothelialization of the injured arteries (elderly versus young, 14±4% versus 36±5%; $P<0.001$; Figure 1A and 1B). Fluorescent microscope revealed that the transplanted EPCs were incorporated at sites of injury, however, less frequently with EPCs from the elderly than EPCs from young people (Figure 1C). Flow cytometry analyses of single-cell suspension made from the injured vessel fragments confirmed fewer DiI-labeled EPCs in mice transplanted with EPCs from the elderly ($P<0.01$; Figure 1D). Furthermore, transplantation of late EPCs from the elderly also displayed a lower degree of reendothelialization and less EPC incorporation than transplantation of late EPCs from young subjects (Figure S5). These results confirmed that EPCs from the elderly have a reduced reendothelialization capacity that is associated with a reduced EPC incorporation in the injured vessels.

CXCR4/JAK-2 Signaling Is Required for EPC Migration and Adhesion and EPC-Mediated Reendothelialization

To understand the molecular mechanism responsible for the impaired reendothelialization capacity of EPCs derived from the elderly, we examined the CXCR4 signaling in EPCs, because CXCR4 is key to homing and retention of EPCs at sites of arterial injury, therefore contributing to the reendothelialization.[26,27] Surprisingly, Western blotting analyses

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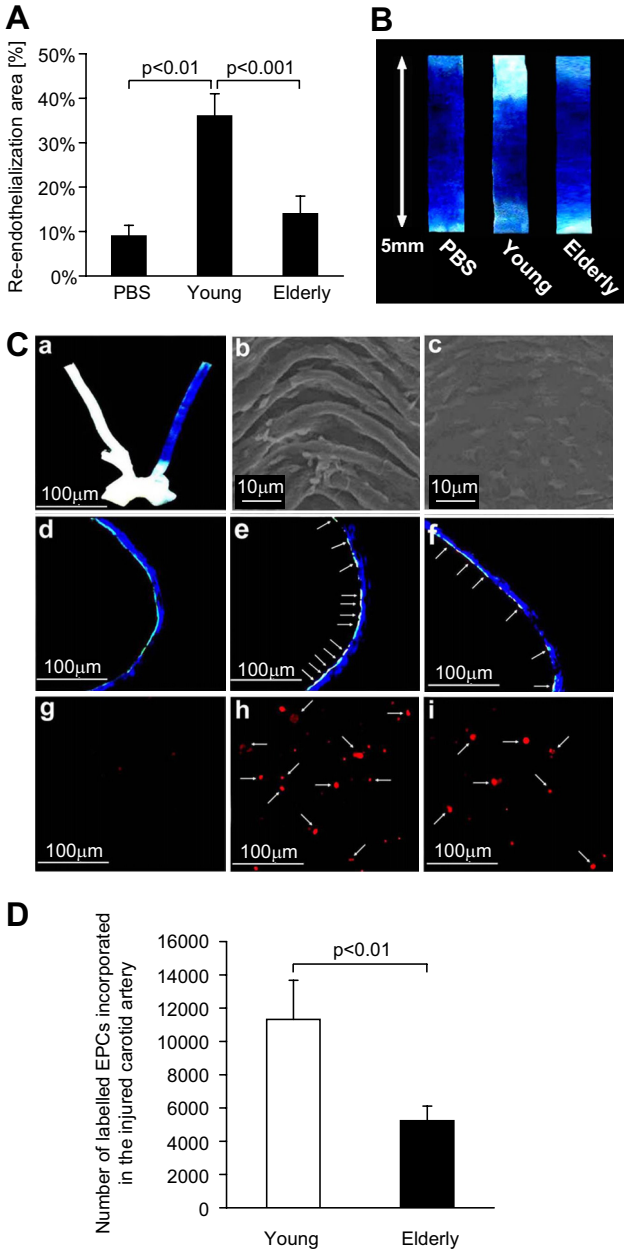

Figure 1. Aging endothelial progenitor cells (EPCs) display a reduced reendothelialization capacity. **A** and **B**, Quantification (**A**) and representative photographs (**B**) of reendothelialization areas of the injured carotid arteries in nude mice 3 days after carotid denudation surgery plus IV injection of PBS or EPCs (5×10^5 cells) cultured from young people or the elderly (n=10 per group). **C** and **D**, Assessments of the transplanted EPCs incorporated in the injured vessels. **C**, **a**, light photograph of injured (stained with Evans blue) and contralateral uninjured carotid artery; **b** and **c**, electron microscope pictures of injured (**b**) and contralateral uninjured carotid arteries (**c**); **d** through **f**, cross-section and (**g** through **i**) en face view of the contralateral uninjured carotid arteries (**d** and **g**) and injured carotid arteries (**e**, **f**, **h**, and **i**) showing chloromethylbenzamido-DiI–labeled EPCs (red) attached to injured endothelium (green) in mice receiving young (**e**) or elderly EPCs (**f**). Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue, **d** through **f**). Shown are representatives of 3 independent experiments. **D**, Fragments of injured carotid arteries were isolated, digested, and made into single cell suspension, and the DiI(+) EPCs were quantified by flow cytometry (n=5 per group). Error bars represent SEM.

indicated that CXCR4 expression did not differ between EPCs from the young and the elderly (Figure 2A); however, the CXCR4 phosphorylation (at serine 339) was reduced in EPCs from elderly (Figure 2B). Because JAK-2 is a well-established downstream target of CXCR4 signaling, we investigated whether the functional status of JAK-2 is altered in EPCs from the elderly. Basal ($P<0.01$) and the SDF-1–induced ($P<0.001$) JAK-2 phosphorylations were significantly lower in EPCs from the elderly than EPCs from young subjects (Figure 2C). Importantly, both short hairpin RNA–mediated knockdown of CXCR4 or pretreatment of EPCs with JAK-2 inhibitor AG490 significantly attenuated EPC migration and adhesion activities and the EPC-mediated reendothelialization (Figure S6). Collectively, these results suggest that the CXCR4/JAK-2 signaling is essential for normal EPC functions, and the reduced reendothelialization capacity of EPCs from the elderly is at least partially related to the reduced CXCR4/JAK-2 signaling.

Shear Stress Enhances the Function and Reendothelialization Capacity of EPCs From the Elderly

We then investigated the effect of in vitro shear stress on the functions of cultured EPCs. Exposure of EPCs to 15 dyne/cm^2 of shear stress for 12 or 24 hours (Figure S7A through S7C) or exposure of EPCs for 12 hours to 10, 15, or 25 dyne/cm^2 of shear stress (Figure S7D and S7E) significantly increased EPC migration toward SDF-1, adhesion to tumor necrosis factor-α–prestimulated HUVECs, and adhesion in flow on fibronectin (Figure S8) in both young and elderly EPCs ($P<0.05$) and abrogated the differences between the 2 groups. In addition, the migration and adhesion activities of EPCs derived from HUVECs and late EPCs from the elderly were also enhanced by in vitro shear stress treatment (Figure S9). To determine the effect of shear stress on EPC-mediated reendothelialization in vivo, EPCs were exposed to 15 dyne/cm^2 of shear stress for 12 hours and then intravenously injected (separately) into nude mice 3 hours after surgical carotid artery denudation. Pretreatment with shear stress enhanced the reendothelialization capacity of both young and elderly EPCs, however, to a greater extent in elderly EPCs (Figure 3A and 3B). Furthermore, in vitro shear stress treatment also significantly enhanced the reendothelialization capacity of late EPCs from the elderly and endothelial-derived EPCs derived from HUVECs (Figure S10).

Shear Stress Upregulates CXCR4 Expression and Enhances SDF-1/CXCR4-Mediated JAK-2 Phosphorylation

We then investigated whether the enhancement of reendothelialization capacity of EPCs by shear stress treatment was related to CXCR4 signaling. Exposure of EPCs to 15 dyne/cm^2 of shear stress for 6, 12, or 24 hours or exposure of EPCs for 12 hours to 5, 10, 15, and 25 dyne/cm^2 of shear stress led to a significant increase in CXCR4 mRNA (Figure S11A and S11B), total (Figure S11C and S11D) and surface CXCR4 protein (Figure 3C and 3D), and phosphorylated surface CXCR4 protein (Figure 3E and 3F; $P<0.05$) but did not alter CD31, kinase insert domain receptor, von Willebrand factor,

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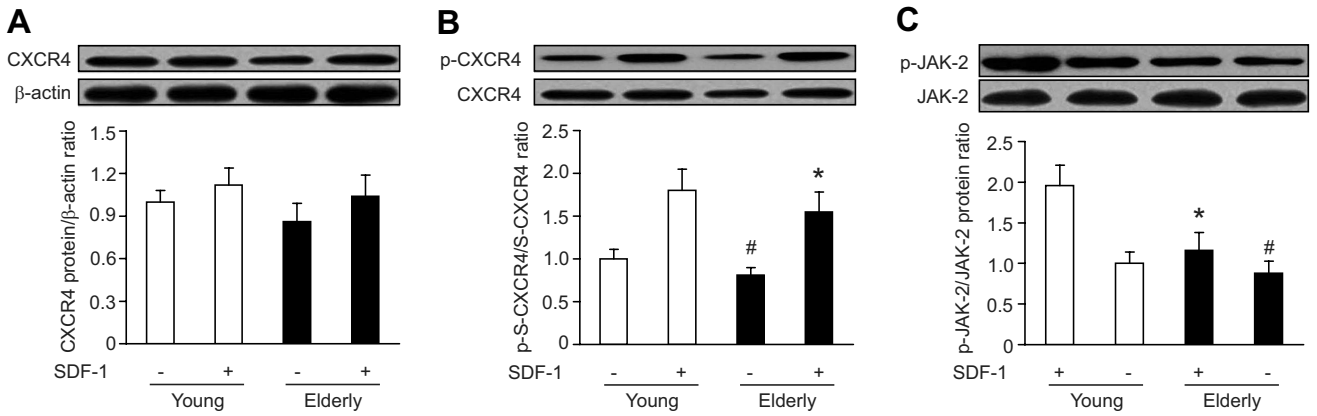

Figure 2. Effect of aging on the CXC chemokine receptor 4 (CXCR4)/Janus kinase 2 (JAK-2) signaling of endothelial progenitor cells (EPCs). Representative photographs (**top panels**) and quantitative analyses (**bottom panels**) of CXCR4 (**A**), phospho-CXCR4 (at Serine 339; **B**), and phospho (p)-JAK-2 (**C**) in the cultured EPCs. (*$P<0.01$ vs young EPCs with SDF-1, #$P<0.05$ vs young EPCs without SDF-1; n=10 per group).

and CD14 expression in EPCs (Figure S12). Furthermore, our results indicated that CXCR4 protein expression in the late EPCs from the elderly (Figure S13A) and in EPCs derived from HUVECs (Figure S13B) was also increased after exposure to 15 dyne/cm^2 of shear stress for 12 hours.

There was no significant difference in basal JAK-2 phosphorylation between the shear stress-treated EPCs and EPCs cultured under static condition; however, the SDF-1–stimulated increase of JAK-2 phosphorylation was significantly greater in shear stress-treated EPCs than in EPCs cultured under static condition ($P<0.01$; Figure 4A), and this difference was diminished by CXCR4 knockdown or by pretreatment of the cells with JAK-2 inhibitor AG490 ($P<0.01$; Figure 4B), suggesting a dependency on the SDF-1/CXCR4 signaling. Interestingly, the combination of JAK-2 inhibition and CXCR4 knockdown did not produce an additive effect. Furthermore, shear stress also potentiated SDF-1–induced JAK-2 phosphorylation in late EPCs from the elderly (Figure S13C).

Shear Stress Enhances the Reendothelialization Capacity of EPCs From the Elderly via CXCR4/JAK-2 Signaling

Next, we investigated whether shear stress-induced upregulation of CXCR4/JAK-2 signaling contributes to the enhancement of EPC functions. Lentivirus short hairpin RNA–mediated CXCR4 knockdown or preincubation of EPCs with JAK-2 inhibitor AG490 significantly attenuated the in vitro migration and adhesion ($P<0.05$; Figure 4C and 4D) and in vivo reendothelialization capacity of the shear stress-treated EPCs from both the young and the elderly ($P<0.01$; Figure 4E and 4F) and abrogated the difference between the 2 groups of cells. In contrast, transduction of EPCs with scrambled short hairpin RNA lentiviral particles had no effect on these functional parameters. Furthermore, CXCR4 knockdown or AG490 treatment also attenuated the shear stress-mediated functions in endothelial-derived EPCs and late EPCs from the elderly (Figure S14). Collectively, these results suggest that shear stress enhances the reendothelialization capacity of EPCs from the elderly through the CXCR4/JAK-2 signaling pathway.

Discussion

The major findings of the present study are as follows: (1) in vitro migration and adhesion activities and in vivo reendothelialization capacity of EPCs in the elderly are significantly reduced; (2) the CXCR4 phosphorylation and CXCR4/JAK-2 signaling are impaired in EPCs from aging populations; and (3) shear stress ameliorates the functional defects of EPCs in the elderly and enhances their reendothelialization capacity by augmenting CXCR4/JAK-2 signaling. Collectively, our study demonstrates for the first time that the functional state of CXCR4 and CXCR4/JAK-2 signaling is critical to the function of EPCs from aging populations and that ex vivo treatment of EPCs with shear stress can be used as an effective approach to enhance the reendothelialization capacity of EPCs.

It has been known that advancing age is associated with a reduction in the number and function of EPCs in humans.[11–13,15–17,19,29] Here we found that EPC migration toward SDF-1 and adhesion to the tumor necrosis factor-α–activated endothelial cells are specifically impaired. Furthermore, EPCs from the elderly exhibited a significantly reduced reendothelialization capacity in the injured carotid artery compared with EPCs from the young subjects, which suggests a reduction of endogenous repair capacity in the vasculature with aging.

CXCR4 is crucial for EPC homing to the local vascular bed to mediate reendothelialization.[26–28,30,31] Given the close association between CXCR4 signaling and EPCs targeted repair for endothelium, we hypothesized that disturbance in CXCR4 signaling is related to the impaired EPC function in the elderly. We show that SDF-1–induced phosphorylations of CXCR4 and JAK-2 are significantly lower in EPCs from the elderly than in EPCs from the young. Furthermore, CXCR4 knockdown and JAK-2 inhibition abrogate the in vitro function and in vivo reendothelialization capacity of EPCs. These data indicate that CXCR4/JAK-2 signaling is critical to the function of EPCs, and decreased phospho–JAK-2 level is, at least in part, related to the reduction in EPC-mediated endothelial repair capacity with aging. Therefore, a novel approach that enhances the CXCR4/JAK-2

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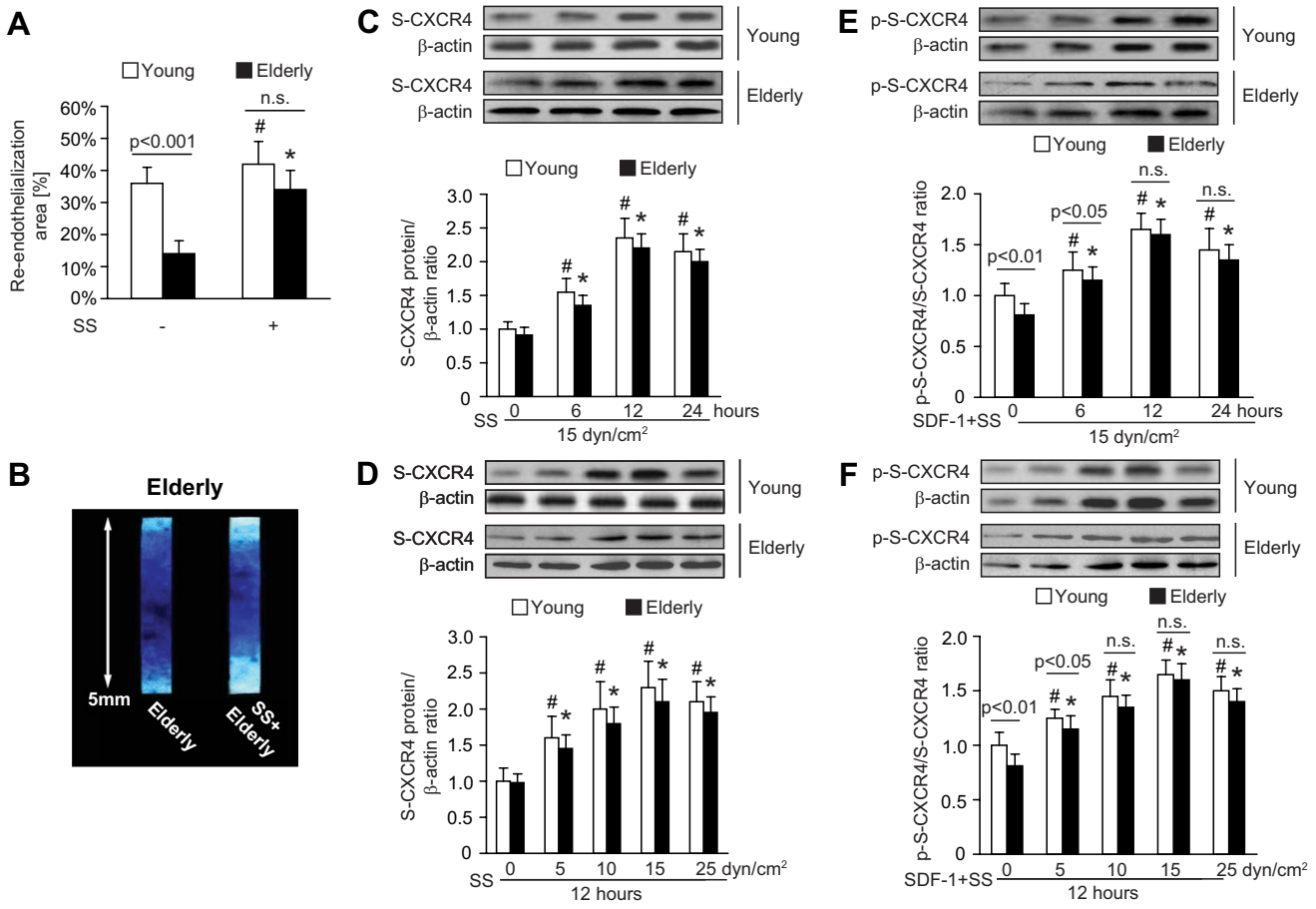

Figure 3. Shear stress enhances in vivo reendothelialization capacity and upregulates CXC chemokine receptor 4 (CXCR4) expression of endothelial progenitor cells (EPCs). **A**, Quantitative analyses and (**B**) representative photographs of reendothelialization areas (white) of carotid arteries 3 days after denudation injury plus IV injection of EPCs with treatment by 15 dyne/cm^2 of shear stress for 12 hours. (#$P<0.05$ vs static young EPCs, *$P<0.001$ vs static elderly EPCs; n=10 per group). **C** and **E**, Representative photograph (**top panels**) and quantification (**bottom panels**) of surface CXCR4 (**C**) and phosphorylated surface CXCR4 (**E**) by Western blotting in EPCs treated with 15 dyne/cm^2 of shear stress for the indicated length of time (#$P<0.05$ vs static young EPCs; *$P<0.05$ vs static elderly EPCs; n=10 per group). **D** and **F**, Representative photograph (**top panels**) and quantification (**bottom panels**) of surface CXCR4 (**D**) and phosphorylated surface CXCR4 (**F**) in EPCs treated for 12 hours with the indicated force of shear stress. (#$P<0.05$ vs static young EPCs; *$P<0.05$ vs static elderly EPCs; n=10 per group). Error bars represent SEM. SS indicates shear stress. □, young; ■, elderly.

signaling may improve the endothelium-reparative potential of EPCs and restore the integrity and homeostasis of the vascular endothelium in the elderly.

We found that shear stress exerts beneficial effects on human EPCs for endothelial protection.[21–24] Previous studies from our laboratory demonstrate that shear stress regulates the expression of endothelial NO synthase, Cu/Zn superoxide dismutase, and tissue plasminogen activator in human EPCs and that transplantation of shear stress-treated EPCs onto the prosthetic vascular grafts leads to the formation of a bioactive endothelial monolayer associated with significant inhibition of thrombosis formation.[22–24] However, whether shear stress regulates CXCR4 signaling was unclear. In this study, we hypothesized that treatment of EPCs with shear stress may enhance CXCR4/JAK-2 signaling, contributing to EPC-mediated reendothelialization after arterial injury. We showed that shear stress upregulates CXCR4 expression and phosphorylation, as well as the responsiveness of EPCs to SDF-1/CXCR4-mediated JAK-2 phosphorylation. This augmentation in shear stress-induced CXCR4/JAK-2 signaling in the elderly EPCs parallels with the enhanced in vitro migration and adhesion activities and in vivo reendothelialization capacity and can be abrogated by CXCR4 knockdown or JAK-2 inhibition. These findings indicate that shear stress is an effective means to modify the biological phenotype of EPCs derived from the elderly and to facilitate EPC-mediated endothelial repair, adding a novel insight into the molecular mechanism of the shear stress on the regulation of EPC biological phenotype related to endothelial repair in humans.

The findings presented in this study have strong clinical implications. Aging results in imbalance of injury and repair in the endothelium and requires a higher efficiency of repair to maintain endothelial homeostasis. Our study clearly shows the dysfunctional properties of EPCs with aging, suggesting a negative impact of aging on the endothelial injury-and-repair balance. The identification of the CXCR4/JAK-2 signaling pathway downregulated with aging may provide molecular targets for the development of treatments to enhance the potential of endogenous EPCs and/or to increase the functional capacity of transplanted cells. The present study

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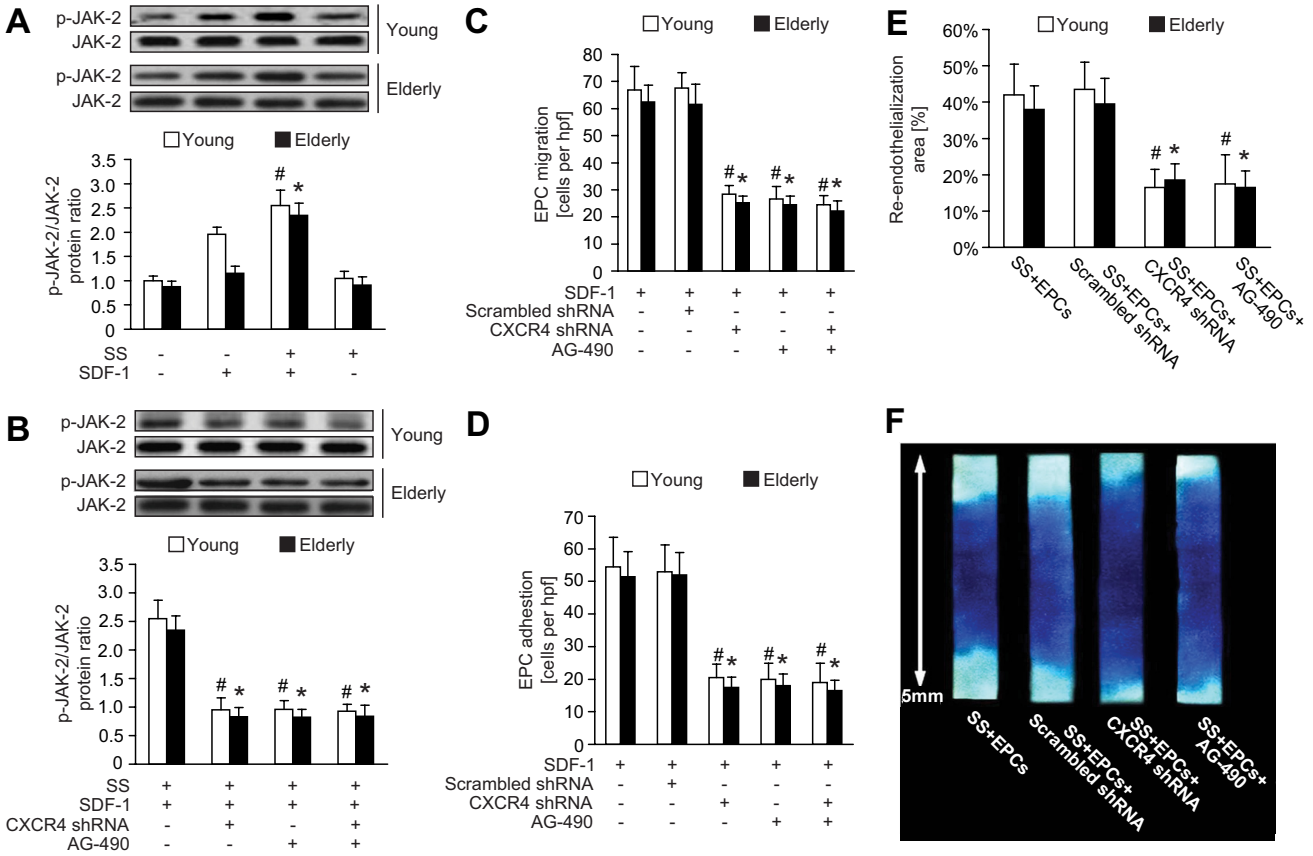

Figure 4. Blockade of CXC chemokine receptor 4 (CXCR4)/Janus kinase 2 (JAK-2) signaling attenuates shear-stress (SS)-mediated enhancement of in vitro function and in vivo reendothelialization capacity of endothelial progenitor cells (EPCs). **A** and **B**, Representative photograph (**top panels**) and quantification (**bottom panels**) of phosphorylated JAK-2 in EPCs (n=10 per group). **C**, Quantification analysis of EPC migration toward SDF-1 (#$P<0.05$ vs SS-treated young EPCs without short hairpin RNA [shRNA] transduction/AG-490 incubation; *$P<0.05$ vs SS-treated elderly EPCs without shRNA transduction/AG-490 incubation; n=10 per group). **D**, Quantification analyses of EPC adhesion to the tumor necrosis factor (TNF)-α prestimulated human umbilical vein endothelial cells (HUVECs; #$P<0.05$ vs SS-treated young EPCs without shRNA transduction/AG-490 incubation; *$P<0.05$ vs SS-treated elderly EPCs without shRNA transduction/AG-490 incubation; n=10 per group). **E** and **F**, Quantification (**E**) and representative photographs (**F**) showing reendothelialization areas of carotid arteries 3 days after denudation injury plus IV injection of EPCs pretreated with CXCR4-shRNA, scrambled-shRNA or AG490. (#$P<0.01$ vs SS-treated young EPCs; *$P<0.01$ vs SS-treated elderly EPCs; n=10 per group). Error bars represent SEM. Hpf indicates high-power field. □, young; ■, elderly.

provides evidence that shear stress other than direct stimulation of its natural ligand SDF-1 can be used to restore the CXCR4/JAK-2 molecular signaling pathway and enhance the endogenous endothelium-reparative capacity of EPCs, which constitutes an important cell-based therapeutic strategy to facilitate EPC function and improve repair capacity in the aging populations.

Perspectives

The present study demonstrates that increasing age leads to impaired reendothelialization capacity of EPCs that is at least partially related to the diminished CXCR4/JAK-2 signaling. Shear stress, therefore, may be a novel approach for EPC-based endothelial repair in the elderly by increasing CXCR4/JAK-2 signaling.

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Disclosures

None.

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