Cleaved High-Molecular-Weight Kininogen Accelerates the Onset of Endothelial Progenitor Cell Senescence by Induction of Reactive Oxygen Species

Jihong Dai, Xuemei Zhu, Mervin C. Yoder, Yi Wu, Robert W. Colman

Objective—Cleaved high-molecular-weight kininogen (HKa), an activation product of the plasma kallikrein-kinin system, inhibits endothelial cell functions. We questioned whether HKa affects the function of endothelial progenitor cells (EPCs) and accelerates their senescence.

Methods and Results—Treatment with HKa for 2 weeks markedly inhibited the formation of large colonies and proliferation of EPCs on collagen surfaces, whereas HKa did not affect collagen-mediated EPC adhesion and survival. Concomitantly, treated EPCs displayed flattened and giant cell morphological changes and formation of intracellular vacuoles. As determined by acidic β-galactosidase staining, HKa increased senescent EPCs by 2- and >3-fold after culture for 1 and 2 weeks, respectively. In addition, HKa suppressed the telomerase activity of EPCs. HKa concentration-dependently increased the generation of intracellular reactive oxygen species (ROS) and markedly upregulated p38 kinase phosphorylation and prosenescence molecule p16INK4a expression. SB203580, a p38 inhibitor, attenuated the level of HKa-enhanced p16INK4a expression. Either quenching of ROS or inhibition of p38 kinase prevented HKa-induced EPC senescence.

Conclusion—HKa accelerates the onset of EPC senescence by activating the ROS–p38 kinase–p16INK4a signaling cascade. This novel activity of HKa points out the likelihood of HKa serving as an endogenous inducer of EPC senescence.

Key Words: aging ■ reactive oxygen species ■ vascular biology ■ endothelial progenitor cells ■ kininogen

Endothelial progenitor cells (EPCs) are precursors to mature endothelial cells that display distinct characteristics.1 These cells have the ability to be mobilized to the site of vascular injury or tissue ischemia, and they differentiate into mature endothelial cells, promoting reendothelialization and neovascularization. Thus, EPCs play an important role in endothelial cell and vessel maintenance. However, in patients with atherosclerosis and cardiovascular diseases, the number and function of EPCs are negatively correlated with the atherosclerotic risk factors, which may contribute to vascular dysfunction.2 For example, the frequency of circulating EPCs is reduced 50% in patients with coronary artery disease, and their EPCs display an impaired migratory response.3 Moreover, the clinical administration of EPCs to patients with cardiovascular diseases has had limited efficacy, whereas in animal models, EPCs successfully restore endothelial function and enhance angiogenesis after tissue ischemia.4 Very likely, EPCs are targets of endogenous angiogenic inhibitors elaborated in the setting of atherosclerosis.4 Therefore, understanding the factors that affect EPC function and number is important, because it could improve the specific therapies to ultimately correct EPC dysfunction and prevent progression of atherosclerosis.

The plasma kallikrein-kinin system (KKS) consists of the proteins factor XII, prekallikrein, and high-molecular-weight kininogen (HK).5 This system displays multiple physiological and pathophysiologic activities, such as blood pressure adjustment, modulation of thrombosis, and regulation of endothelial cell function and angiogenesis. Plasma HK is a major component of the KKS and is responsible for the association of this system with the cell surface. The membrane surface of endothelial cells is an important site for the assembly and activation of the KKS.5 Activation of the KKS is triggered in vivo by tissue destruction or by thrombus development6 and results in cleavage of HK by kallikrein into 2-chain HK (HKa). Unlike HK, HKa exposes its domain 5 to the surface on cleavage, thereby acquiring a function of antiadhesion.7 This antiadhesive property enables HKa to inhibit endothelial cell proliferation and to induce endothelial apoptosis on extracellular proteins.8,9

On the basis of these investigations, we examined in this study whether HKa targets EPCs and regulates their function and number. Our data demonstrate that HKa inhibited clonogenic and proliferative capacities of EPCs and accelerated the onset of EPC senescence. HKa significantly increased intra-
cells were counted were scored positive for proliferation. a fluorescence microscope, and wells in which 2 or more endothelial was changed every 2 days. On day 14, each well was examined under collagen containing EGM-2 medium. After 24 hours, individual wells were examined under a fluorescence microscope, and the wells that contained only 1 cell were scored in the assay. The cell culture medium were examined for the phenotypic and functional validation analysis.

EPC Preparation
In this study, those EPCs referred to as endothelial colony-forming cells were isolated from peripheral blood, and their progenitor cell capacities were validated as previously described. To be defined as an EPC, endothelial colony-forming cells were examined for evidence of clonal proliferative potential, endothelial cell surface phenotype, and in vitro capillary lumen formation. In brief, after informed consent was obtained in accordance with the Declaration of Helsinki, human blood was collected from healthy volunteer donors. After 1:1 dilution with Hanks’ buffered salt solution, blood was overlayed onto Ficoll-Paque and centrifuged at 740g for 30 minutes. Buffy coat mononuclear cells were collected and resuspended in complete endothelial growth culture medium-2 (EGM-2, Cambrex) with additives (Bullet Kit) provided by the manufacturer and 10% fetal bovine serum. The cells were cultured in a 6-well tissue culture plate precoated with type 1 collagen (BD Biosciences) at 37°C. Colonies appearing between 5 and 22 days of culture were identified as a well-circumscribed monolayer of cobblestone-appearing cells. EPCs at early passages (passage 1 to 3) were examined for the phenotypic and functional validation analysis.

Lentiviral Transduction of EPCs and Single-Cell–Based Colony Formation Assay
Lentivirus expressing enhanced green fluorescent protein (EGFP) was generated as previously described. The initial mononuclear cells on collagen-coated plates were infected with the supernatant containing lentivirus-expressing EGFP diluted 1:1 with complete EGM-2. The culture medium was replaced every 2 days. After 3 weeks, EGFP-positive EPCs (EGFP-EPCs) were observed under fluorescence microscope and sorted by fluorescence cytometry. A single EGFP-EPC was placed in 1 well of a 96-well tissue culture plate precoated with type 1 collagen containing EGM-2 medium. After 24 hours, individual wells were examined under a fluorescence microscope, and the wells that contained only 1 cell were scored in the assay. The cell culture medium was changed every 2 days. On day 14, each well was examined under a fluorescence microscope, and wells in which 2 or more endothelial cells were counted were scored positive for proliferation.

Data Analysis
Unless stated otherwise, the results shown are from a single experiment representative of at least 3 separate experiments. The data were calculated as average±SEM from experiments done at least 3 times and statistically analyzed by the Student t test (2 groups only) or 1-way ANOVA and the Student-Newman-Keuls test (multiple groups). Differences with probability values below 0.05 were considered significant.

The Supplemental Data (available online at http://atvb.ahajournals.org) provide a complete description of materials and methods used for reagents, cell proliferation assay, measurement of mRNA expression by reverse transcription–polymerase chain reaction, immunoblot analysis and adhesion assay, senescence-associated β-galactosidase activity assay, terminal deoxynucleotidyl transferase dUTP nick-end labeling–based apoptosis detection assay, intracellular ROS production, and telomerase activity assay.

Results
HKa Suppresses Clonogenic Capacity of EPCs
As described in our previous studies, EPCs have been isolated and expanded ex vivo from adult peripheral blood mononuclear cells. We analyzed the clonogenic capacity of EPCs by using a single-cell assay previously described. Within the first week after isolation, mononuclear cells were cultivated in the EGM-2 containing lentivirus-expressing EGFP, followed by culture in EGM-2. A single colony of EPCs uniformly expressing EGFP appeared in most wells (Figure 1A), and the colonies were collected for subsequent studies. To test whether HKa affects the clonal expansion potential of EPCs, a single-cell suspension of EGFP-EPCs was seeded into 96-well culture plate...
precoated with collagen. Because it has been observed that more than 30% of plasma HK is cleaved in patients with sepsis and autoimmune diseases,14,15 in this study we chose concentrations of HKa between 30 and 100 nmol/L, 5% to 15% of plasma concentration (660 nmol/L). The culture medium EGM-2 was replaced every 2 days. After culture for 14 days, EGFP-EPCs formed large colonies (Figure 1B), and 85% of colonies contained more than 200 cells (large colonies; Figure 1C). However, in the presence of 50 nmol/L HKa, the percentage of large colonies was markedly reduced to 6% (P<0.001, Figure 1B and 1C), indicating that HKa strongly inhibits clonogenic capacity of EPCs.

HKa Inhibits EPC Proliferation Without Induction of Apoptosis
HKa inhibition of EPC colony formation suggested that HKa suppresses proliferation of EPCs. We therefore examined the effect of HKa on proliferative capacity of EPCs using a 5-bromo-2'-deoxyuridine incorporation assay. Figure 2A indicates that treatment of EPCs with HKa for 72 hours significantly inhibited vascular endothelial growth factor–stimulated 5-bromo-2'-deoxyuridine incorporation into EPCs, and the inhibition was concentration-dependent, whereas the significant inhibitory effect of HKa was detectable only at 100 nmol/L after treatment for 48 hours. In contrast to its significant effects on proliferation, HKa at the same concentrations did not inhibit EPC adhesion to collagen (Figure 2B), suggesting that HKa inhibition of EPC proliferation does not result from an antiadhesive activity. Because HKa induces apoptosis of differentiated endothelial cells on vitronectin-coated surfaces,9 we examined the effect of HKa on induction of EPC apoptosis. Figure 3 indicates that HKa did not induce EPC apoptosis on collagen surfaces, although its effect on induction of EPCs apoptosis on vitronectin-coated plates was significant. Therefore, HKa inhibition of EPC colony formation and proliferation is not subject to antiadhesive activity and induction of apoptosis.

HKa Accelerates the Onset of EPC Senescence and Suppresses Telomerase Activity
Reduction of EPCs in number and activity has previously been associated with EPC senescence.16 The common fea-
HKα Increases Intracellular ROS Production, Contributing to EPC Senescence

It has been well documented that cell senescence is tightly associated with intracellular ROS production. We tested whether HKα exposure increases intracellular ROS in EPCs using the 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) labeling assay. As shown in Figure 5A, in a concentration-dependent manner, HKα significantly increased H2DCF-DA oxidation level in exposed EPCs. Therefore, we hypothesized that HKα accelerates EPC senescence via ROS production, and performed an inhibition assay using the ROS scavengers Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) and N-acetylcyesteine (NAC). MnTBAP is a cell-permeable superoxide dismutase mimetic and peroxynitrite scavenger. NAC is an efficient free radical scavenger and contributes to production of other antioxidant species. Treatment with either 100 μmol/L NAC or 10 μmol/L MnTBAP significantly attenuated the increase in ROS production in EPCs treated with 100 nmol/L HKα for 12 hours (Supplemental Figure I). Moreover, 100 μmol/L NAC or 10 μmol/L MnTBAP significantly reduced both the percentage of senescent cells and ROS production in EPCs treated with 50 nmol/L HKα for 14 days (Figure 5B and 5C). These observations suggest that HKα generation of ROS is involved in the acceleration of EPC senescence.

HKα Upregulates p38 Kinase Phosphorylation and p16INK4a Expression

To investigate the mechanism for HKα acceleration of EPC senescence, we measured the levels of p38 kinase activation and senescence molecule p16INK4a expression, which are downstream of ROS generation in the process of cellular senescence. Immunoblotting analysis indicated that during the 7-day experimental period, phosphorylation of p38 kinase was increased by HKα at 30 and 100 nmol/L (Figure 6A). Concomitantly, HKα upregulated p16INK4a expression at the protein level (Figure 6A). Figure 6B shows an increase in p16INK4a mRNA expression in HKα-treated EPCs. To determine the connection between p38 kinase activation and p16INK4a expression, EPCs were treated with SB203580, a specific p38 kinase inhibitor. Figure 6C demonstrates that 10 μmol/L SB203580 markedly suppressed HKα-induced p16INK4a expression, suggesting that HKα upregulates p16INK4a expression via its activation of p38 kinase. Moreover, 10 μmol/L SB203580 significantly attenuated HKα-mediated EPC senescence (Figure 6D).

Figure 4. HKα accelerates the onset of EPC senescence and suppresses telomerase activity. A, Representative photomicrographs of SA-β-gal-positive EPCs (magnification, ×40). EPCs were cultured in the presence of 0.1% BSA or 50 nmol/L HKα for 14 days. Culture medium was replaced every 2 days. SA-β-gal activity of EPCs was analyzed as described in Materials and Methods. Scale bars represent 50 μm. Arrows indicate intracellular vacuoles. B, Percentage of senescent cells. EPCs were cultured in the presence of 0.1% BSA (open columns) or 50 nmol/L HKα (gray columns) for the indicated period of time. The number of SA-β-gal-positive EPCs (senescent cells) was counted (n=6). *P<0.05, **P<0.01 vs BSA. C, Effect of HKα on EPC telomerase activity. After EPCs were cultured in the presence of 0.1% BSA or 50 nmol/L HKα for 14 days, telomerase activity was measured as described in Materials and Methods. Data are mean±SEM, n=4. *P<0.001 vs BSA.

HKα increases an increase in cellular vacuoles. To determine whether HKα induces EPC senescence, we measured the activity of acidic β-galactosidase, referred as senescence-associated-β-galactosidase (SA-β-gal). Although a small portion of EPCs were positive for SA-β-gal staining after culture for 14 days (Figure 4A and 4B), in the presence of 50 nmol/L HKα, the majority of EPCs became positive for SA-β-gal staining (Figure 4A). Moreover, EPCs treated with 50 nmol/L HKα displayed a unique flattened and enlarged morphology and formed intracellular vacuoles (Figure 4A). Figure 4B demonstrates that HKα treatment significantly resulted in an increase in SA-β-gal-positive cells, 17.2±2.6% vs 52.3±3.7% on day 7 and 22.3±2.7% vs 85.5±7.9% on day 14. Acceleration of the onset of EPC senescence is known to be critically influenced by the level of telomerase activity, which elongates telomeres, thereby counteracting telomere length reduction induced by each cell division. Therefore, we tested whether HKα treatment was capable of regulating telomerase activity in EPCs. Figure 4C demonstrates that treatment of EPCs with 50 nmol/L HKα for 14 days markedly diminished telomerase activity by >60% (P<0.005), serving as additional evidence for HKα acceleration of EPC senescence.

HKα increases intracellular vacuoles (Figure 4A). Figure 4B demonstrates that HKα flattened and enlarged morphology and formed intracellular vacuoles. To determine whether HKα treatment resulted in an increase in SA-β-gal-positive cells, 17.2±2.6% vs 52.3±3.7% on day 7 and 22.3±2.7% vs 85.5±7.9% on day 14. Acceleration of the onset of EPC senescence is known to be critically influenced by the level of telomerase activity, which elongates telomeres, thereby counteracting telomere length reduction induced by each cell division. Therefore, we tested whether HKα treatment was capable of regulating telomerase activity in EPCs. Figure 4C demonstrates that treatment of EPCs with 50 nmol/L HKα for 14 days markedly diminished telomerase activity by >60% (P<0.005), serving as additional evidence for HKα acceleration of EPC senescence.
Discussion

Our current study reveals for the first time that HKa accelerates the onset of EPC senescence and induces typical morphological changes of EPC senescence. Consistently, HKa inhibited telomerase activity and the clonogenic and proliferative capacities of EPCs. HKa treatment increased intracellular ROS generation, and the quenching of ROS by NAC and MnTBAP prevented HKa-induced EPC senescence. Additionally, HKa increased p38 kinase activity and p16INK4a expression in EPCs, suggesting that ROS may contribute to the induction of p16INK4a expression.

Figure 5. HKa stimulates generation of ROS in EPCs. A, H$_2$DCF-DA oxidation in EPCs. As described in Materials and Methods, EPCs were preincubated with 10 μmol/L H$_2$DCF-DA for 30 minutes and then treated with or without HKa for 12 hours. H$_2$DCF-DA oxidation was analyzed by flow cytometry. The data are expressed as the mean channel fluorescence (MCF) of H$_2$DCF-DA and represent the average of 3 separate experiments (±SEM).*P<0.05, **P<0.001. B and C, Effect of NAC and MnTBAP on HKa-induced EPC senescence (B) and ROS production (C). EPCs were cultured in the absence or presence of 50 nmol/L HKa for 14 days. As indicated, EPCs in separate groups were also treated with 0.1% BSA (−), 100 μmol/L NAC, or 10 μmol/L MnTBAP. Percentage of senescent cells was calculated and depicted (B), and intracellular ROS level are expressed as a percentage of controls. *P<0.001 vs BSA; n=3.

Figure 6. HKa increases p38 kinase activity and p16INK4a expression in EPCs. A, EPCs were cultured in the presence of 0.1% BSA (lane 1), 30 nmol/L HKa (lane 2), or 100 nmol/L HKa (lane 3) for 14 days. A representative immunoblot of phosphorylated p38 kinase and p16INK4a (p16) level in EPCs is shown. The blots for p38 kinase and β-actin served as loading control. B, Reverse transcription–polymerase chain reaction (RT-PCR) analysis of p16INK4a (p16) mRNA expression in EPCs, which were cultured with or without 50 nmol/L HKa for 14 days. A representative agarose gel image from 3 experiments is shown. C, EPCs were cultured in the presence or absence of 50 nmol/L HKa with or without 10 μmol/L SB203580 for 14 days. The level of p16 expression was analyzed by immunoblotting. A representative result from 3 experiments is shown. The blot for β-actin served as loading control. D, EPCs were cultured as described in C. The percentage of senescent EPCs was calculated by SA-β-gal assay. *P<0.01; n=3.
ence. HKα treatment enhanced p38 kinase phosphorylation and upregulated p16\(^{INK4a}\) expression in the exposed EPCs. Inhibition of p38 kinase by SB203580 attenuated HKα-increased p16\(^{INK4a}\) expression and EPC senescence. Thus, this study demonstrates novel activities of HKα in regulating several key elements of EPC biology.

Aging is associated with an increased risk for atherosclerosis, and insufficient repair of damaged vascular walls by a diminished number or dysfunction in EPC is one of many possible causes.\(^2\) A reduction in EPC number and activity has been associated with EPC senescence.\(^3\) Because senescence limits the ability of EPCs to sustain ischemic tissue repair, a full characterization of the pathophysiological factors leading to EPC senescence, as well as the related underlying mechanisms, is clearly important. Our current study demonstrating HKα acceleration of EPC senescence not only expands our understanding of the KKS activation in the regulation of vascular biology but also reveals a potential novel endogenous inducer of EPC senescence.

The in vivo activation of the KKS and cleavage of HKα has been widely detected in numerous pathophysiological conditions, such as thrombosis,\(^6\) arthritis,\(^20\) inflammatory bowel disease,\(^21\) vasculitis,\(^22\) sepsis,\(^23\) systemic amyloidosis,\(^24\) and preeclampsia.\(^25\) However, our understanding of how the KKS activation products initiate and regulate downstream effects remains elusive. It has been well documented that HKα inhibits the function of differentiated endothelial cells, such as human umbilical vein endothelial cells in vitro, as well as perturbing in vivo angiogenesis.\(^26,27\) In this study, our results have provided new evidence that HKα targets EPCs and accelerates the onset of their senescence. The plasma concentration of HK is 660 nmol/L. In patients with sepsis and autoimmune diseases, more than 30% of plasma HK was cleaved.\(^14,15\) Because the minimal concentration of HKα that significantly induced EPC senescence was 30 nmol/L (data not shown), the circulating levels of HKα in the pathological settings could affect EPC function. This HKα-mediated effect was associated with profoundly impaired EPC clonal expansion potential and resulted in a low overall proliferative capacity of the EPC progeny. Because HK is localized at sites of vascular injury, such as atherosclerotic lesions,\(^28\) it might become cleaved over the abundant negative-charged surfaces to release HKα. The observation that HKα potently inhibited the clonogenic capacity of EPCs suggests that HKα is possibly involved in the vascular dysfunction by blocking EPC aggregation and expansion. Because HKα inhibition of EPC function is not dependent on its antiadhesion activity, HKα in plasma may attack circulating EPCs. Although this study provides the first evidence for prosenescent activity of HKα, whether the blockade of HKα cleavage prevents EPC senescence remains to be determined using an in vivo model.

EPC senescence can be triggered by a variety of factors, such as proinflammatory cytokines, DNA damage, hyperoxia, and hyperglycemia.\(^18\) All these factors increase oxidative stress, by which they induce EPC senescence and suppress telomerase activity.\(^16\) The mechanism by which HKα accelerates the onset of senescence of EPCs seems to be tightly associated with ROS production. NAC and MnTBAP, which quench ROS, significantly attenuated HKα-induced EPC senescence (Figure 5). A previous study has shown that HKα inhibits Akt phosphorylation and endothelial nitric oxide synthase phosphorylation,\(^29\) which may inhibit the production of nitric oxide (NO). Although NO prevents endothelial cell senescence,\(^30\) the NO donor S-nitrosothioproline did not prevent HKα-accelerated onset of EPCs senescence (data not shown). Thus, HKα regulation of EPC senescence appears independent of the NO pathway. Accumulation of ROS in most cell types is associated with high expression of p16\(^{INK4a}\), which leads to the arrest of the cell cycle at the G\(_1\) phase and accelerates senescence of cells.\(^31\) The expression of p16\(^{INK4a}\) increases with age and contributes to age-dependent stem and progenitor cell senescence.\(^32\) It can be deduced that accumulative ROS exposure, caused by various atherosclerotic risk factors, may also increase the expression of p16\(^{INK4a}\) in EPCs, contributing to EPC senescence. We found that HKα increased expression of p16\(^{INK4a}\) at the mRNA and protein level (Figure 6), demonstrating that HKα accelerates EPC senescence by the regulation of p16\(^{INK4a}\) expression. We previously have found that p38 kinase is responsible for mediating the effect of HKα in other cells.\(^33\) Our observations in this study indicate that HKα enhances phosphorylation of p38 kinase, and the inhibition of p38 kinase prevented both HKα-induced p16\(^{INK4a}\) expression and EPC senescence. Therefore, HKα-induced ROS seems to act through p38 kinase to upregulate prosenescent molecule p16\(^{INK4a}\) expression, which may further regulate telomerase activity and result in EPC senescence.

In conclusion, the present study demonstrates that HKα accelerates the onset of EPC senescence by the activation of the ROS/p38 kinase/p16\(^{INK4a}\) signaling cascade. This activity of HKα reveals a novel link between KKS activation and vascular dysfunction, and it expands our understanding of the additional pathophysiological activities of this system. Although it remains to be determined whether and how HKα inhibition of EPCs is involved in the disordered vascular remodeling that occurs in vivo, our novel recognition that components of plasma KKS modulate EPC function allows for focused determination of the pathophysiologic activities of plasma KKS in atherosclerosis in future studies.

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Disclosures
None.

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Supplemental Figure I.

Attenuation by NAC or MnTBAP of HKa-induced ROS production in EPCs.

After EPCs were labeled with 10 µM H$_2$DCF-DA for 30 minutes, they were exposed to 100 nM HKa with PBS (-), 100 µM NAC or 10 µM MnTBAP for 12 hours. Mean channel fluorescence of H$_2$DCF-DA was analyzed by flow cytometry. Results are expressed as percent of control. (*$P$ < 0.01, v.s. PBS; n=3).
Supplemental Materials and Methods:

**Reagents**

Unless otherwise specified, all reagents were obtained from Sigma (St Louis, MO).

**Cell Proliferation Assay**

Proliferation of EPCs was analyzed by the incorporation of 5-bromo-2′-deoxyuridine (BrdU). EPCs (600 cells/cm²) in EGM-2 were seeded in collagen-coated 96-well culture plates precoated with collagen and cultured at 37°C with 5% CO₂ for 5 hours. The medium was replaced with fresh medium containing 2% serum, 10 ng/ml VEGF, 25 µM ZnCl₂ and 10 µM BrdU, with or without HKα (Enzyme Research Laboratories). The culture medium was replaced every 24 hours. Subsequently, the BrdU incorporation into cells was determined with a Cell Proliferation ELISA Kit (Roche Diagnostics).

**Measurement of mRNA Expression by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

Total RNA was isolated from EPCs using Trizol® reagent (Invitrogen). For RT-PCR, primer sequences used were as follows: p16 sense, CAACGCACCAGAATAGTTACG; p16 antisense, AGCACCAGACGCTGTC; β-actin sense, AGCGAGCATCCCCAAAGTT; β-actin antisense, GGGCACGAGGCTCATCATT. RNA was used as template in a SuperScript
One-Step RT-PCR reaction (Invitrogen), performed according to the manufacturer's instructions.

**Immunoblot Analysis**

Cell lysates were prepared in a buffer containing 25 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, and protease inhibitor cocktail (Roche Molecular Biochemicals). Lysates were assayed for total protein concentration (BCA assay, Pierce), and 30 µg of clarified extract were resolved on a 4-20 % gradient SDS-polyacrylamide gel. Proteins were transferred to PVDF membranes (Millipore) and incubated at 4°C overnight, and detected using the following antibodies: rabbit polyclonal anti-p16 (c-20, Santa Cruz), rabbit anti-p38 MAPK monoclonal antibody and phosphorylation-specific monoclonal antibody for p38 MAPK (Thr180/Tyr182) (Cell Signaling Technology). Horseradish peroxide-conjugated secondary antibodies were used for detection by enhanced chemiluminescence.

**Adhesion Assay**

The 96-well culture plates were coated with matrix proteins at 37°C for 4 hours and then blocked with 0.3% BSA in PBS. Cells were plated at a density of 3.0 × 10^4/well and incubated at 37°C for 2 hours. After unattached cells were washed away with PBS, the attached cells were quantified by a Cell Titer AQeuous analysis kit from Promega (Madison, WI).

**Senescence-associated β-galactosidase Activity Assay**

Cell senescence was evaluated by measurement of senescence-associated
β-galactosidase (SA-β-gal) activity using a β-Galactosidase Staining Kit (BioVision). Briefly, EPCs were washed in PBS and fixed for 15 min at room temperature with fixative solution. After incubation with the staining solution mixture overnight at 37°C, the cells were observed under a microscope for development of blue color. The absolute numbers of SA-β-galactosidase-positive cells were counted.

**Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-based Apoptosis Detection Assay**

After treatments, EPCs were fixed in 2% paraformaldehyde in PBS for 10 minutes and washed 3 times with PBS, and permeabilized with 0.2% Triton X-100. The cells were stained using the Dead/End Fluorometric apoptosis detection system (Promega), following the instructions of the manufacturer, producing fluorescein TUNEL staining and DAPI nuclear counterstaining. Merged images were generated by dual scanning at 488 nm and 543 nm with a Leica SP1 confocal microscope and TUNEL-positive or -negative cells were counted in 6 random fields per well in a blinded fashion.

**Intracellular ROS Production**

The oxidation-sensitive dye 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCF-DA) (Molecular Probes) was used for measurement of intracellular ROS generation. Briefly, after EPCs were preincubated with 10 μM H₂DCF-DA at 37°C for 20 minutes, they were challenged with HKa. Incubation was stopped by washing with PBS and FACS analysis was performed immediately on a FACS Calibur flow cytometer (Becton Dickinson).
**Telomerase activity assay**

Telomerase activity was measured using the quantitative TeloTAGGG Telomerase PCR ELISA<sup>PLUS</sup> kit (Roche Molecular Biochemicals). EPCs (5 x 10<sup>4</sup>) were plated in a 60 mm well in EGM-2 medium containing 10% FBS and cultured for the indicated time periods. After treatment, EPCs were harvested in 200 µl 1 x lysis reagent and the telomerase activity in cell lysates was measured according to the manufacturer’s instructions.