Stem Cell Factor Attenuates Vascular Smooth Muscle Apoptosis and Increases Intimal Hyperplasia After Vascular Injury


Objective—Stem cell factor (SCF) through its cognate receptor, the tyrosine kinase c-kit, promotes survival and biological functions of hematopoietic stem cells and progenitors. However, whether SCF/c-kit interactions attenuate VSMC apoptosis induced by vascular injury has not been thoroughly investigated.

Methods and Results—VSMCs were stimulated with serum deprivation and H2O2 to induce apoptosis. The transcription of c-kit mRNA and the expression of the c-kit protein by VSMCs were estimated by Q-polymerase chain reaction and Western blotting, respectively. The interactions of SCF and c-kit were investigated by in vitro and in vivo experiments. In vitro, H2O2 stimulation significantly induced apoptosis of VSMCs as evidenced by the 3- and 3.2-fold increases of cleaved caspase-3 compared with those in the control group by Western blot and flow cytometric analyses, respectively (P<0.01). Stimulation of apoptosis also caused 3.5- and 9-fold increases in c-kit mRNA transcription and protein expression, respectively, by VSMCs compared with those in the control group. Administration of SCF (10 to 1000 ng/mL) significantly lowered the amount of cleaved caspase-3 in H2O2-treated VSMCs (P<0.01). Specifically, SCF exerted this effect through activating Akt, followed by increasing Bcl-2 and then inhibiting the release of cytochrome-c from the mitochondria to the cytosol. In vivo, the mouse femoral artery was injured with a wire in SCF mutant (Sl/Sl'P), c-kit mutant (W/Wv), and colony control mice. In colony control mice, confocal microscopy demonstrated that the wire-injury generated a remarkable activation of caspase-3 on medial VSMCs, coinciding with upregulation of c-kit expression. The wire-injury also caused an increase in the expression of SCF on surviving medial VSMCs and cells in the adventitia. The upregulated c-kit expression in the vessel wall also facilitated homing by circulating SCF+ cells. Compared with colony control mice, vascular injury in SCF mutant and c-kit mutant mice caused a higher number of apoptotic VSMCs on day 14 and a lower number of proliferating cells, and resulted in significantly less neointimal formation (P<0.01) on day 28.

Conclusions—The interactions between SCF and the c-kit receptor play an important role in protecting VSMCs against apoptosis and in maintaining intimal hyperplasia after vascular injury. (Arterioscler Thromb Vasc Biol. 2007;27:000-000.)

Key Words: apoptosis • c-kit tyrosine kinase • intimal hyperplasia • restenosis • stem cell factor

Neointimal formation, with resultant vascular remodeling, is a unifying pathological event complicating chronic atherosclerosis, restenosis, and transplant arteriopathy, and remains the major limiting factor for the long-term efficacy of vascular interventions, such as angioplasty and coronary artery bypass graft surgery.1,2 VSMC proliferation coincides with apoptosis in vessels undergoing angioplasty.3 The consequences of early-onset apoptosis in medial VSMCs after vascular injury and late apoptosis in the neointima have not been fully investigated. Although it plays an important role in limiting neointimal growth and the subsequent intimal hyperplasia, it may also exacerbate neointima formation at later time points by provoking a greater wound-healing response in an effort to overcome the cellular deficit.4,5

Stem cell factor (SCF, Steel Factor) through its cognate receptor, the tyrosine kinase c-kit,6 promotes survival,7 proliferation,8 mobilization,9 and adhesion10 of hematopoietic stem cells and their progenitors. Recently, the existence of
this system has also been demonstrated in the vasculature.\textsuperscript{11,12} However, the role of its function in atherosclerosis is poorly understood. In this study, we hypothesized that early-onset apoptosis in medial VSMCs after vascular injury activates the SCF/c-kit system, which protects VSMCs from apoptosis and contributes to over-growth of the neointima.

Methods
An expanded Materials and Methods section is available in the online data supplement at http://atvb.ahajournals.org.

Animals Studies
Wild-type C57BL/6, W/W\textsuperscript{v} WBB6F1 hybrid strain, c-kit mutant mice, colony control WBB6F1 (+/+), Sl/SI\textsuperscript{a} (Steel-Dickie; WCB6F1 hybrid strain, SCF mutant mice), and colony control WCB6F1 (+/) mice were purchased from Jackson Labs (Bar Harbor, Me) and were used for the vascular injury studies. All procedures involving experimental animals were performed in accordance with protocols approved by the institutional committees for animal research of Toronto General Hospital, Mount Sinai Hospital, and Chang Gung Memorial Hospital and were conducted according to guidelines of the American Physiological Society.

Mouse Femoral Artery Wire-Injury Model
Femoral arterial injury was induced by inserting a straight spring wire (0.38 mm in diameter, No. C-SF-15-15, Cook) for more than 5 mm toward the iliac artery.\textsuperscript{13}

Cell Culture
Human aortic smooth muscle cells were purchased from Smartec Scientific (Cascade Biologics) and grown in 231 medium with smooth muscle cell growth supplement, plus 50 U/mL penicillin and 50 \( \mu \text{g/mL} \) streptomycin in a humidified atmosphere of 5\% CO\textsubscript{2}. More than 97\% of the cultured cells were VSMCs as confirmed by immunostaining with a monoclonal \( \alpha \)-smooth muscle actin (\( \alpha \)SMA) antibody. Cells used for the experiments were in the third to fifth passages and were 80\% confluent. To produce apoptosis by serum deprivation and H\textsubscript{2}O\textsubscript{2}, cells were washed with PBS, the medium was replaced with serum-free medium with H\textsubscript{2}O\textsubscript{2} (000 \( \mu \text{mol/L} \), and the cells were incubated and harvested at the indicated time points. Smooth muscle progenitors,\textsuperscript{15} endothelial progenitors,\textsuperscript{16} and late-outgrowth endothelial cells (OECs)\textsuperscript{16} were also cultured using the standard methods mentioned previously.

Bone Marrow Transplantation Model
Recipient FVB mice at 8 weeks of age were lethally irradiated with a total dose of 9.0 Gy. eGFP transgenic mice (FVB background) that ubiquitously expressed enhanced GFP were used as the donors (Level Biotechnology Inc., Taipei, Taiwan).\textsuperscript{17} After irradiation, the recipient mice received unfractionated bone marrow cells (5\times10\textsuperscript{6}) from eGFP mice by tail vein injection. At 8 weeks after the injection, these mice received wire injury to the femoral artery. Repopulation by eGFP-positive bone marrow cells was measured by flow cytometry to be 95\%.

Results
Apoptosis Stimulation Upregulates c-kit mRNA Transcription and Protein Expression
After H\textsubscript{2}O\textsubscript{2} stimulation, although there was no significant change in the total caspase-3 amount, caspase-3 was transiently activated as indicated by the amount of cleaved caspase-3 (Figure 1A). The activation of caspase-3 peaked at 0.5 hour and then gradually returned back to the baseline. Apoptosis stimulation also induced a 25-fold increase in the number of apoptotic cells as indicated by Annexin-V\textsuperscript{+}PI\textsuperscript{+} cells (Figure 1B). By RT-PCR and Q-PCR (Figure 1C and 1D, respectively), VSMCs had a low baseline level of c-kit mRNA transcription. In response to apoptosis stimulation, the transcription of c-kit mRNA increased by approximately 5.5-fold (1 hour after H\textsubscript{2}O\textsubscript{2} administration). By Western blotting, c-kit protein expression was present at the baseline and significantly increased after apoptosis stimulation in human VSMCs (Figure 1E). Immunofluorescent staining also demonstrated substantial c-kit expression on Annexin-V\textsuperscript{+} apoptotic VSMCs (supplemental Figure I). In the in vivo animal model, wire injury activated caspase-3 throughout the entire vessel and stimulated c-kit and cleaved caspase-3 coexpression on medial VSMCs at different time points (Figure 1F). Because the anti-c-kit antibody used in this study (sc-168) maps the cytoplasmic domain of the c-Kit protein (amino acids 925 to 975),\textsuperscript{18,19} the cytoplasmic staining pattern was observed in the majority of our immunofluorescence images.

SCF Attenuates the Activation of Apoptosis
In vitro, SCF (10 to 1000 ng/mL) was administered in VSMCs treated with H\textsubscript{2}O\textsubscript{2}. SCF significantly attenuated the amount of cleaved caspase-3 in a dose-dependent manner (Figure 2A). As estimated by flow cytometry, SCF (100 ng/mL) caused a 3-fold decrease in the number of VSMCs with activated caspase-3 (Figure 2B). Using annexin-V to estimate the number of VSMCs undergoing apoptosis, SCF (1000 ng/mL) also significantly lowered the number of apoptotic VSMCs from 24.7\%\pm3.5\% to 11.8\%\pm2.7\% (\( P<0.01 \)) (Figure 2C). To clarify whether SCF also has effects on proliferation in VSMCs with apoptotic stimulation, a proliferation assay was performed and showed that SCF significantly increased VSMC proliferation only at a high concentration (1000 ng/mL) (Figure 2D). The in vivo experiments revealed that after the femoral artery had been injured by the wire, SCF expression was greatly upregulated in the adventitia and in surviving medial VSMCs, providing a direct source of SCF for rescuing injured VSMCs (Figure 2E). The upregulated transcription of SCF mRNA at injured sites was also quantified by Q-PCR, which demonstrated a 3.5-fold increase in the SCF mRNA amount on injured vessels 4 days after wire-injury, compared with the baseline.

C-kit-Positive Cells Help SCF-Positive Cell Homing
Mouse femoral arteries were injured with a wire and then subjected to immunostaining at indicated time points. On days 1 and 3, although remarkable SCF expression was noted in cells in the adventitia, no cells had adhered to the surface of the injured vessel wall (Figure 3A). On day 7 to 9 after vascular injury, many SCF\textsuperscript{+} cells had accumulated on the surface of the injured vessel wall, close to the sites with c-kit protein expression (Figure 3A and 3B; supplemental Figure II). In the in vivo experiments, SCF expression was investigated in a variety of bone marrow–derived progenitor cells, including smooth muscle progenitors, endothelial progenitors, OECs, and human aortic endothelial cells (supplemental Figure IIIA through IIID). All these cells strongly expressed SCF, as demonstrated by immunofluorescent staining and Western blot analysis (supplemental Figure IIIE).
Progenitor cell homing involves cellular adhesion. As mentioned above, OECs strongly expressed SCF. In the adhesion assay, apoptotic VSMCs greatly increased the adhesion of OECs, an interaction that was blocked by administration of the anti-SCF antibody (Figure 3C). Furthermore, a modified Boyden chamber was used to assess the chemotactic potential of c-kit+ cells to SCF+/H11001 OECs. 

H2O2-stimulated VSMCs, compared with unstimulated VSMCs, induced significantly more OEC migration (Figure 3D). A significant abolishment of this migratory effect by an SCF blockade further supported the chemotactic ability of c-kit+/H11001 cells to SCF+/H11001 cells. Similar findings were also repeated with SCF+/H11001 smooth muscle progenitors (data not shown). To clarify the related mechanisms, our data demonstrated that H2O2 stimulation upregulated the expression of SCF by VSMCs (supplemental Figure IVA). In a dose-dependent manner, SCF significantly increased the production of VEGF (VEGF-R2) and SDF-1α (CXCR4) on OECs20 and smooth muscle progenitor cells,21 respectively, the migratory ability of these cells toward VSMCs undergoing apoptotic stress substantially increased.

In the in vivo model of wire-induced femoral artery injury in wild-type mice reconstituted with BM cells expressing eGFP (BMTGfp→Wild mice), results showed that smooth muscle progenitor cells expressed SCF in the early phase after attaching to the injured vessel wall but became SCF-negative when they were mature in the neointima (supplemental Figure IIIF and IIIG). Although it is not fully understood how these cells adapt to this environment and how differentiation is coordinated, these cells had high potential to contribute to intimal hyperplasia.

**SCF Attenuates Apoptosis Through the Akt-Bcl-2 Pathway**

Although there was no significant change in the total Akt amount, treatment with SCF transiently activated Akt, which peaked at 0.5 hour, followed by a significant increase in the...
amount of Bcl-2 (Figure 4A). The control group revealed a significant increase in the amount of cytosolic cytochrome-c which was maintained at a higher level in response to apoptosis stimulation compared with the baseline (Figure 4B). However, in the SCF treatment group, cytosolic cytochrome-c levels significantly decreased and were maintained at a very low level compared with the control group.

Apoptosis in SCF and c-kit Mutant Mice

W/W^v^ mice are compound heterozygotes of a null c-kit mutation (W), and the W-viable (W^v) allele exhibits reduced kinase activity and represents the severest c-kit mutants that survive gestation. Similarly, Sl/Sld mice are compound heterozygotes of a null SCF mutation (Sl) and the Steel-Dickie (Sld) mutation, which lacks mSCF, and represent the severest SCF mutants that survive gestation. Thus, W/W^v^ mice have a relative deficiency of c-kit kinase activity, although the affected cells express normal to elevated levels of the c-kit receptor and Steel-Dickie mice have a complete deficiency of membrane-bound SCF.

Intimal hyperplasia was significantly decreased in both SCF mutant and c-kit mutant mice compared with colony control mice (Figure 5A). As estimated by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining, the number of apoptotic cells tended to be higher on the vessel wall in both SCF and c-kit mutant mice compared with the media and adventitia of colony control mice on day 7 after vascular injury (Figure 5C). On day 14, the number of apoptotic cells on the entire vessel wall was significantly higher in both SCF mutant and c-kit mutant mice compared with colony control mice (Figure 5B and 5C). On the other hand, the number of proliferating cells on the vessel wall was significantly decreased in both SCF mutant and c-kit mutant mice compared with colony control mice (Figure 5D).
wall 7 days after wire injury was significantly lower in SCF mutant and c-kit mutant mice compared with colony control mice (Figure 5D).

Discussion

This study demonstrated that the SCF/c-kit system plays a critical role in the mechanisms of vascular remodeling, namely intimal hyperplasia, in response to the early-onset apoptosis in medial VSMCs after injury. The transcription of c-kit mRNA and the expression of c-kit protein by VSMCs were significantly upregulated in response to apoptotic stimulation. The increased c-kit expression on VSMCs not only helps SCF exert its effects through protecting VSMCs from apoptosis and increasing VSMC proliferation but also facilitates the homing process of SCF-positive cells, which contribute to intimal hyperplasia. Furthermore, SCF attenuated the apoptosis of VSMCs through the Akt–Bcl-2 pathway. The SCF/c-kit system orchestrated exacerbation of neointimal formation after vascular injury at least in part by attenuating VSMC apoptosis and provoking a greater wound healing response to overcome the cellular deficit.

Stem cell factor (SCF, Steel Factor) through its cognate receptor, the tyrosine kinase c-kit, has been shown to promote survival, proliferation, mobilization, and adhesion of a variety of hematopoietic progenitors. Recently, this system was also demonstrated in the vascular system, although its role is still not fully understood. Matsui et al found that SCF improves a variety of biological functions and survival of human umbilical vein endothelial cells. Hollenbeck et al showed that this system is expressed by and may affect VSMCs through an autocrine pathway. In the present study, we demonstrated that this system also exerts its effect on the exacerbation of intimal hyperplasia through upregulating c-kit expression on apoptotic VSMCs, protecting
VSMCs from apoptosis, and increasing VSMC proliferation and homing SCF⁺ cells. In addition, associated mechanisms and the signaling pathway are also provided.

The consequences of early-onset apoptosis in medial VSMCs after vascular injury have not been fully investigated. VSMC apoptosis has been demonstrated in atherosclerosis and in restenotic lesions after angioplasty. In animal models of balloon vascular injury, medial VSMC apoptosis and subsequent cell loss were observed soon after the injury. The molecular mechanisms of vascular cell apoptosis remain to be elucidated, and the role of VSMC apoptosis in vascular remodeling is still a matter of controversy. It has been proposed that VSMC apoptosis prevents proliferative vascular disease, because forced induction of VSMC apoptosis by gene modification results in a reduction of vascular lesions. In contrast, it has also been postulated that vascular cell apoptosis plays a role in the development of vascular lesions, because exuberant balloon-induced apoptosis results in enhanced neointimal formation. As proposed by the current study, this phenomenon can be attributed, at least in part, to the SCF/c-kit system. Our results demonstrated that an apoptosis-stimulating stress substantially upregulated the transcription of c-kit mRNA and the synthesis of c-kit protein. The c-kit receptor is a member of the type III cytokine receptor tyrosine kinase family. This family of cytokine receptors also encompasses the c-fms receptor, the platelet-derived growth factor receptors, and the flk-2/flt-3 receptor. Specifically, our findings suggest that SCF exerts its antiapoptotic effect through c-kit tyrosine kinase and then Akt, followed by a remarkable increase in the amount of intracellular Bcl-2, leading to a substantial inhibition of the release of cytochrome-c from the mitochondria to the cytosol (Figure 4C). Through this pathway, SCF significantly attenuates the activation of caspase-3 and eventual cellular apoptosis.

On the other hand, the upregulated expression of c-kit receptors on VSMCs undergoing apoptosis not only activated this SCF/c-kit system but also helped SCF-positive cells home in on injured vascular sites. These SCF-positive cells provided a substantial amount of SCF to rescue VSMCs from apoptosis and also substantially contributed to the formation of neointima. The concentration of SCF in normal human serum is, on average, 3.3 ng/mL. Our data showed that SCF already exerts its effects at similar concentrations in a dose-dependent manner at higher concentrations. Local SCF concentrations at injured vascular sites, as provided by locally accumulated SCF-expressing cells or bone marrow–derived progenitor cells, are expected to be much higher than serum levels. Consistent with this notion, our findings revealed that smooth muscle progenitor cells, late-outgrowth of endothelial cells, endothelial progenitor cells, and aortic endothelial cells all express SCF. These findings are in line with the new paradigm that bone marrow–derived progenitor cells contribute to intimal hyperplasia after vascular injury, as shown extensively in the literature. However, how the fate of these SCF-positive cells is decided in this microenvironment still remains to be elucidated.

The mouse femoral artery wire-injury model adopted in the present study represents a severe vascular injury model. Although the extent of vascular injury is considerably less severe in human angioplasty, in the era of extensive vascular stent intervention, similar stresses on vessel walls may exist as atherosclerotic plaques are pushed outward. In this study, we also took advantage of SCF-mutant and c-kit mutant mice to gain further support for our hypothesis. In SCF-mutant mice, deficiencies of both mSCF and sSCF disrupted the ability of the SCF/c-kit system to rescue injured VSMCs as indicated by extensive apoptotic events throughout all three layers of the injured vessel wall. Their deficiencies in SCF also substantially attenuated the contribution of SCF-positive cells to the formation of neointima. Although SCF is not deficient in c-kit mutant mice, the lack of appropriate c-kit tyrosine kinase signaling also led to remarkable apoptotic processes on the injured vessel wall, especially on the vascular media and adventitia. Through these mechanisms, these mutant mice ended up with significantly less intimal hyperplasia compared with colony control mice. The effect of...
SCF in stimulating VSMC proliferation may also contribute to growth of the neointima. However, SCF exerts this effect only at high concentrations.

In summary, we herein demonstrate a novel way by which the SCF/c-kit system works on vascular remodeling processes. These findings illustrate that the SCF/c-kit interaction is a very complicated process. To attenuate intimal hyperplasia or atherosclerotic processes, our work provides a rationale for testing directed therapies aimed at interrupting the SCF/c-kit pathway in patients undergoing vascular interventions such as a bypass graft and angioplasty.

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Materials and Methods

Animals Studies

Wild-type C57BL/6, W/W\textsuperscript{v} (WBB6F1 hybrid strain, c-kit mutant mice), colony control WBB6F1 (+/+), Sl/Sld (Steel-Dickie; WCB6F1 hybrid strain, SCF mutant mice), and colony control WCB6F1 (+/) mice were purchased from Jackson Labs (Bar Harbor, ME) and were used for the vascular injury studies. All procedures involving experimental animals were performed in accordance with protocols approved by the institutional committees for animal research of Toronto General Hospital, Mount Sinai Hospital, and Chang Gung Memorial Hospital and were conducted according to guidelines of the American Physiological Society.

Mouse Femoral Artery Wire-Injury Model

Transluminal mechanical injury of the femoral arteries was carried out using a dissecting microscope. Briefly, either the left or right femoral artery was exposed by blunt dissection. The accompanying femoral nerve was carefully separated and the femoral vein was isolated from the artery.\textsuperscript{1} Femoral arterial injury was induced by inserting a straight spring wire (0.38 mm in diameter, No. C-SF-15-15, Cook, Bloomington, IN) for more than 5 mm toward the iliac artery. At indicated time points, femoral arteries were excised and fixed in 10% formalin. Paraffin-embedded sections were used for immunohistochemical staining. Femoral arteries fixed in OCT compound (TissueTek, Tokyo, Japan) were used for the immunofluorescence studies.

Cell Culture
Human aortic smooth muscle cells were purchased from Smartec Scientific (Cascade Biologics) and grown in 231 medium with smooth muscle cell growth supplement, plus 50 U/ml penicillin and 50 μg/ml streptomycin in a humidified atmosphere of 5% CO₂. More than 97% of the cultured cells were VSMCs as confirmed by immunostaining with a monoclonal α-smooth muscle actin (αSMA) antibody. Cells used for the experiments were in the third to fifth passages and were 80% confluent. To produce apoptosis by serum deprivation and H₂O₂, cells were washed with PBS, the medium was replaced with serum-free medium with H₂O₂ (800 μM), and the cells were incubated and harvested at the indicated time points. Smooth muscle progenitors, endothelial progenitors, and late-outgrowth endothelial cells (OECs) were also cultured using the standard methods mentioned previously.

**Adhesion Assay and Co-culture Experiments**

To assess the extent of adhesion of SCF-expressing cells to VSMCs undergoing apoptosis, human aortic VSMCs were grown to confluency on 24-well plates, and then were starved and treated with H₂O₂ for 24 h to induce apoptosis. Human OECs, which strongly expressed SCF, were labeled with 5-chloromethylflourescein diacetate (Molecular Probes, Eugene, OR), treated with or without the SCF antibody (1 μg/ml, Cedarlane), added to the VSMC-coated wells for 30 min at 37°C, and then washed. Numbers of adherent human OECs were counted at five low-power fields under an FITC filter.
Migration Assay

After being resuspended in EBM-2, $1 \times 10^5$ OECs labelled with 5-chloromethylfluorescein diacetate were placed in the upper chamber of a modified Boyden chamber. The lower chamber contained EBM-2 and VSMCs stimulated with $H_2O_2$. After VSMCs had precipitated in the lower chamber, the migration assay was begun. After 4 h of incubation at 37°C, cells migrating into the lower chamber were manually counted in 3 random microscopic fields.

Confocal Immunofluorescent, Immunohistochemical, and Histological Analyses

Frozen sections and cells were stained with first antibodies (Cy3-conjugated anti-α-SMA, Sigma; cleaved caspase-3, Cell Signaling; SCF and c-Kit (sc-168), Santa Cruz; CD45, PharMingen; Annexin-V, BD Bioscience) followed by incubation with Alexa 647, FITC, or PE-conjugated secondary antibodies. Nuclei were stained with either propidium iodide (Sigma) or Hoechst 33258 (Sigma). Sections were mounted using a Prolong Antifade kit (Molecular Probes) and observed under a confocal microscope (Leica TCS SP2 AOBS). In formalin-fixed, paraffin-embedded sections, the intima/media ratio was measured by hematoxylin and eosin (H&E) staining. TUNEL staining was performed to detect cells undergoing apoptosis. The number of apoptotic cells was counted under high-power fields (HPF). Cell proliferation was estimated with immunohistochemical staining by Ki-67 (a marker of cell proliferation) on vessels 14 days after injury.

Western Blot Analysis

The effects of serum deprivation with $H_2O_2$ on inducing apoptosis in human
VSMCs were investigated. The effect of SCF (10~1000 ng/ml) on attenuating VSMC apoptosis was also determined by Western blotting. Briefly, after culturing with different treatments, VSMC lysates were fractionated through a 4% stacking and 10% running SDS-PAGE gel. The fractionated proteins were transferred to PVDF membranes. Blots were blocked for 1 h at room temperature with blocking buffer (5% nonfat milk in 10 mmol/L Tris pH 7.5, 100 mmol/L NaCl, and 0.1% Tween 20). Blots were then incubated with antibodies for apoptosis signaling (Akt, phosphorylated Akt, Bcl2, and cytochrome c, Cell Signaling), anti-cleaved caspase-3 (Cell Signaling), anti-SCF (Santa Cruz), and anti-c-Kit (Santa Cruz and Cell Signaling) polyclonal antibodies overnight at 4 °C. After washing (2x for 15 min in 1x TTBS), the blots were incubated with HRP–conjugated goat anti-rabbit immunoglobulin antibody for 1 h at room temperature. For determination of cytochrome-c, cytosolic and membrane proteins were separated using a Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (PIERCE) and only the cytosolic protein was used. Visualization was performed using enhanced chemiluminescence. Densitometric analysis of the Western blots was performed using the PDI Imageware System.

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) and Real-Time Quantitative PCR Systems (Q-PCR)

VSMCs and mouse vascular tissues were harvested at the indicated time points. Mouse vascular tissues were homogenized with the MagNA Lyser (Roche Diagnostics). Total cellular RNA was extracted using the Trizol reagent. RT-PCR was performed with the OneStep RT-PCR Kit (Promega). For each reaction, 1 µg of total RNA served as a template. For amplification, primer pairs
specific for human \textit{c-kit} (sense primer, 5'-AGG GAG GTA TGG ACT GGG-3'; and antisense primer, 5'-CAC GGA CAA TTT ATA GAT GC-3') and for mouse SCF (sense primer, 5'- GCG CTG CCT TTC CTT ATG AA -3' and antisense primer, 5'- TAT TAC TGC TAC TGC TGT CA -3') were used. The RT-PCR products of \textit{c-kit} and SCF were 434 and 619 bp, respectively. Reverse transcription was performed at 94 °C for 5 min. For PCR, 28 cycles at 94 °C for 1 min, 60.3 °C for 1 min, and 72 °C for 2 min were used. The RT-PCR products were visualized on 1% agarose gels with ethidium bromide. Q-PCR was performed with the Master Mix of LightCycler® TaqMan Master Kit (Roche Diagnostics, Penzberg, Germany). The reaction sequence included denaturation for 10 min at 95 °C before 45 cycles of denaturation for 10 s at 95 °C, annealing for 30 s at 60 °C, and extension for 1 s at 72 °C. Thermal cycling and collection of fluorescence data were done in a Roche LightCycler Instrument.

**Flow Cytometric Analysis of Apoptosis and Caspase 3 Activity**

Cells were labeled with annexin-V (BD Bioscience) and propidium iodide (PI, Sigma) according to the manufacturer’s instructions. Samples (10^4 events) were analyzed with a flow cytometer (Coulter EPICS), and the distribution of cells was determined with multicycle software (Coulter EPICS XL/XL-MCL System II). Cells labeled with annexin-V^-/PI^- were considered apoptotic cells, and those labeled with annexin-V^-/PI^+ were considered necrotic cells. The number of VSMCs with cleaved caspase-3 was enumerated using the PhiPhiLux-G1D2 kit (OncoImmunin, MD).
**Proliferation Assay**

To determine whether SCF promoted the proliferation of VSMCs with apoptotic stimulation, VSMCs were stimulated with H_2O_2 (800 μM) for 1h, followed by replacing the culture medium containing PDGF (10 ng/ml) or SCF (10 – 1000 ng/ml). An Absolute-S™ Kit was used for measuring cell proliferation by dual color flow cytometry (Chemicon International) in accordance with the manufacturer's directions. The stained cells were analyzed on a Coulter EPICS flow cytometer using appropriate positive and negative controls.

**Bone Marrow Transplantation Model**

Recipient FVB mice at 8 weeks of age were lethally irradiated with a total dose of 9.0 Gy. eGFP transgenic mice (FVB background) that ubiquitously expressed enhanced GFP were used as the donors (Level Biotechnology Inc., Taipei, Taiwan). After irradiation, the recipient mice received unfractionated bone marrow cells (5 x 10^6) from eGFP mice by tail vein injection. At 8 weeks after the injection, these mice received wire injury to the femoral artery. Repopulation by eGFP-positive bone marrow cells was measured by flow cytometry to be 95%.

**Statistical Analysis**

Data were compared by unpaired t-test or ANOVA with Tukey's test. For the data represented as percentages which did not follow a normal distribution of variances, logarithmic transformation was performed prior to analysis. A p value of < 0.05 was considered significant. All data presented in the text and figures are expressed as the mean ± SEM.
REFERENCES


c-kit Annexin-V Nucleus

Fig. I
Fig. II

A M L
A M
M
M L
A A
A

D0
D1
D4
D7
D9

c-kit
SCF
Nuclei
A. Endothelial progenitor cells (in vitro)

B. Outgrowth of endothelial cells (in vitro)

C. Smooth muscle progenitor cells (in vitro)

D. Human aortic endothelial cells (in vitro)

E. Stem cell factor expression

F. Smooth muscle progenitor cells (in vivo)

G. Smooth muscle progenitor cells (in vivo)

Fig. III
Fig. IV
Supplemental figure legends

Supplemental Figure I. F. Immunostaining pattern of c-kit on apoptotic VSMCs.

Supplemental Figure II. Confocal immunofluorescent analysis was performed on femoral arteries at different time points after wire injury (red, SCF; green, c-kit; blue, nuclei). A, adventitia; M, media; L, lumen.

Supplemental Figure III. Bone marrow-derived vascular progenitor cells are SCF-positive. Confocal immunofluorescence images show that SCF was expressed by a variety of vascular progenitor cells, such as early endothelial progenitor cells (EPCs) (A), late-outgrowth of endothelial cells (late EPCs or OECs) (vWF, von Willebrand factor) (B), smooth muscle progenitor cells (SPCs) (α-SMA, α-smooth muscle actin) (C) and human aortic endothelial cells (HAECs) (D). Expression of SCF protein on these cells was confirmed by Western blot (E). PBMNC, peripheral blood mononuclear cell. In vivo, on D7 after wire injury to a femoral artery of bone marrow-reconstituted FVB mice (BMTGfp→Wild mice), some of the bone marrow-derived SPCs (arrow) were α-SMA⁺SCF⁺eGFP⁺ (F). Although on D10, SPCs were α-SMA⁺SCF⁺ (red, α-SMA; yellow, double positive for α-SMA and SCF; blue, nuclei) (G), however, SPCs became SCF⁻ when they matured at later time points (D28) (630×). D7 indicates day 7, and so on. Scale bar = 50 μm.

Supplemental Figure IV. Western blotting demonstrates that apoptosis stimulation with H₂O₂ significantly upregulates expression of the stem cell factor (SCF) by vascular smooth muscle cells (VSMCs) (A). In a dose-dependent manner, 24-h treatment with SCF significantly increased the secretion of VEGF and SDF-1α by VSMCs undergoing apoptotic stimulation (B and C, respectively). VEGF and SDF-1α concentrations in the supernatant were measured by ELISA (R&D). * p < 0.05, ** p < 0.01 compared to VSMCs without stimulation.