Secretoneurin, an Angiogenic Neuropeptide, Induces Postnatal Vasculogenesis

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Background—Induction of postnatal vasculogenesis, the mobilization of bone marrow–derived endothelial progenitor cells and incorporation of these cells into sites of blood vessel formation, is a well-known feature of angiogenic cytokines such as vascular endothelial growth factor. We hypothesized that the angiogenic neuropeptide secretoneurin induces this kind of neovascularization.

Methods and Results—Secretoneurin induced mobilization of endothelial progenitor cells to sites of vasculogenesis in vivo in the cornea neovascularization assay. Progenitor cells were incorporated into vascular structures or were located adjacent to them. Systemic injection of secretoneurin led to increase of circulating stem cells and endothelial progenitor cells. In vitro secretoneurin induced migration, exerted antiapoptotic effects, and increased the number of these cells. Furthermore, secretoneurin stimulated the mitogen-activated protein kinase system, as shown by phosphorylation of extracellular signal–regulated kinase, and activated the protein kinase B/Akt pathway. Activation of mitogen-activated protein kinase was necessary for increase of cell number and migration, whereas Akt seemed to play a role in migration of endothelial progenitor cells.

Conclusions—These data show that the angiogenic neuropeptide secretoneurin stimulates postnatal vasculogenesis by mobilization, migration, and incorporation of endothelial progenitor cells. (Circulation. 2004;110:1121-1127.)

Key Words: angiogenesis ■ endothelium ■ nervous system

The postnatal growth of new blood vessels results from 2 different biological processes, angiogenesis and vasculogenesis. Whereas angiogenesis describes the generation of new vessels by sprouting from the already existing vasculature, vasculogenesis refers to a de novo formation of vessels by circulating endothelial progenitor cells. The process of vasculogenesis was considered to be confined to the embryo until recently, when several groups reported the existence of bone marrow–derived endothelial progenitor cells (EPCs) (the circulating cells were also called circulating endothelial precursor cells [CEPs]). The contribution of these cells in postnatal neovascularization was demonstrated in tumor angiogenesis, hindlimb and myocardial ischemia, and reendothelialization of denuded segments of arterial vessels. Function of these cells is impaired in patients with vascular risk factors such as diabetes or smoking. Recently, the therapeutic potential of these cells was demonstrated in ischemic heart disease as well as in peripheral arterial disease in human patients. Mobilization of these cells is a typical feature of angiogenic cytokines such as vascular endothelial growth factor (VEGF) but was also reported for granulocyte/macrophage colony-stimulating factor (GM-CSF), statins, and erythropoietin.

Secretoneurin is a neuropeptide derived from secretogranin II, an abundant protein in neuroendocrine storage vesicles and a member of the chromogranin/secretogranin family. Recently, we demonstrated that secretoneurin acts as direct angiogenic cytokine in vivo and in vitro, inhibits endothelial cell (EC) apoptosis, stimulates EC proliferation, and activates the mitogen-activated protein kinase (MAPK) system and the Akt pathway.

Because of the angiogenic effect of secretoneurin, we investigated the influence of secretoneurin on postnatal vasculogenesis.

Methods

Secretoneurin Peptide and Antibody

The peptide secretoneurin and the secretoneurin antibody were described in detail before. The peptide was dissolved in PBS.
containing 0.1% bovine serum albumin. This solvent was used as control. Secretoneurin was purchased from Neosystems.

Animal Models

The St Elizabeth’s Medical Center Institutional Animal Care and Use Committee approved all protocols. Anesthesia was performed with 2,2,2-tribromoethanol (880 mmol/kg body wt IP; Sigma-Aldrich).

Mouse Bone Marrow Transplantation Model

Mouse bone marrow transplantation (BMT) models were performed as previously described.7,13 In brief, lethally irradiated FVB/N mice (Jackson Laboratories, Bar Harbor, Me) received bone marrow cells from transgenic Tie2/LacZ mice, which express β-galactosidase encoded by LacZ under the transcriptional regulation of an endothelium-specific gene, Tie2.

Mouse Cornea Neovascularization Model

Pellets containing 300 ng VEGF-165 (Chemicon) or 300 ng secretoneurin were implanted in the corneas of FVB/N mice 4 weeks after BMT as described.13 Corneas were examined by slit-lamp biomicroscopy on postoperative day 7. After the mice were euthanized, dissected corneas were subjected to whole-mount X-Gal staining.13 Tie2/LacZ-positive endothelial lineage cells were counted per cornea. Afterward, cross sections of corneas were stained with isocitrate B4 to identify corneal neovascularization.7

Mouse EPC Culture

Mouse EPC culture was performed as described with slight modifications.7,8,13,14 In brief, peripheral blood mononuclear cells (PBMCs) were isolated from mice injected with secretoneurin (10 μg/d IP for 7 days) or solvent by density gradient centrifugation. Four days after BMT, cells were stimulated with Flt-1 ligand plus 0.5% gelatin (5.106 cells/well of a 24-well plate). EPCs were assayed by culturing with acetylated LDL (aLDL)/DiI (Biomedical Technologies) and fluorescein isothiocyanate (FITC)-conjugated BS-1 lectin (Vector). Double-positive cells per high-power field (HPF) (×100) were counted.

FACS Analysis of Circulating Stem Cells

Murine bone marrow cells and PBMCs of FVB mice (treated with secretoneurin as described above) were isolated by standard procedures. The presence of murine precursor cells was determined by fluorescence-activated cell sorter (FACS) analysis (FACScalibur, Becton Dickinson) by staining with FITC-labeled antibody against Sca-1 (Ly-6A/E) and phycoerythrin (PE)-labeled antibody against c-kit (CD117; all antibodies from Pharmingen, Mississauga, Ontario, Canada). Levels of nonspecific staining were established by parallel analyses of cultures incubated with irrelevant isotype-matched control antibodies.

Human EPC Culture

PBMCs were isolated from human volunteers and cultured on human fibronectin in EBM-2 medium (Clonetics) as described.8–13 EPCs were characterized as described in detail previously.7 Additionally, typical hemangioblastic islets1 in EPC cultures were stained for aLDL/DiI uptake and FITC-conjugated Ulex europaeus lectin (Sigma) binding. Expression of mRNA for endothelial markers KDR (VEGF receptor 2) and CD-31 (platelet–endothelial cell adhesion molecule [PECAM]) by EPCs was assayed by reverse transcription–polymerase chain reaction (RT-PCR) as described.13 The following primers were used: KDR forward: GAA GAC AGG CTA CTG GTC CAT CGT C; KDR backward: GAT CCA CAG GGA TTG CTC CAA CGT A; CD-31 forward: GAG GAC ATT GTG CTG CAA TGT GCT G; CD-31 backward: AAC TTC CGT GTA CGT CAC GTC TGA G.

For determination of cell number, cells were treated in 24-well plates at day 3 of culture with different concentrations of secretoneurin, 1 ng secretoneurin plus anti-secretoneurin antibody, 50 ng/mL VEGF, 10 nmol/L wortmannin (phosphatidylinositol 3 kinase [PI3-kinase] inhibitor), 10 μmol/L PD98059 (MAPK inhibitor) (all from Sigma), or different combinations. After 24 hours, cells were assayed by culturing with acLDL/DiI and FITC-conjugated Ulex europaeus lectin. Double-positive cells per HPF were counted.

For annexin staining, EPCs were starved at day 4 of culture for 12 hours in the presence of 10 ng/mL secretoneurin or solvent. Afterward, cells were stained as described by the manufacturer (Annexin-V FLUOS kit, Roche).

Western Blotting

EPCs were starved for 24 hours, treated with 1 ng secretoneurin or 10 ng secretoneurin for 5, 10, or 20 minutes or 20% fetal bovine serum (positive control), and processed for Western blotting as described.23 In some experiments cells were preincubated with PD98059 or wortmannin 30 minutes before secretoneurin was added.

EPC Chemotaxis Assay

Chemotaxis of EPCs (5x104 cells/well) to gradients of secretoneurin or VEGF (10 ng/mL) was measured as described23 with a microchemotaxis chamber (Neuroprobe) and a 5-μm-pore–sized cellulose nitrate filter (Sartorius). Attractants were either 10 ng/mL VEGF (as positive control) or various concentrations of secretoneurin. After 6 hours at 37°C, the filters were stained with hematoxylin-eosin.23 Migration was quantified by microscopy, measuring the distance from the surface of the filter to the leading front of cells.23 The data are expressed as relative chemotactic index (ratio between distance of migration toward the test attractants and distance of migration toward control medium). The mean migration depth of the leading front in controls was 20 μm.

Statistical Analysis

All results are expressed as the mean±SEM. Statistical comparisons between groups were performed by ANOVA. Probability values <0.05 were considered to denote statistical significance.

Results

In Vivo Experiments

Secretoneurin Mobilizes Bone Marrow–Derived EPCs to Sites of Vasculogenesis

FVB mice were transplanted with bone marrow from Tie2/LacZ donors and subjected to cornea neovascularization assay after 4 weeks by implantation of a pellet containing VEGF (Figure 1a) or secretoneurin (Figure 1b). After 7 days, both cytokines induced an angiogenic response, as shown by slit-lamp biomicroscopy (Figure 1a, 1b, insets), demonstrating vessels originating from the limbus artery and growing toward the cytokine pellet, as also described previously.22 Pellets containing control buffer showed no vessel growth.22 X-Gal–positive, blue cells were abundantly mobilized from bone marrow to the corneal neovascularization and quantitatively comparable for both cytokines (VEGF, 111±25; secretoneurin, 137±32 cells per whole-mount cornea; P=NS; n=3 for each cytokine). Cells appeared to be most densely distributed at the leading front of neovascularization around the cytokine-containing pellet (Figure 1a, 1b).

Corneal cross sections of the area between the limbus artery and the secretoneurin pellet (Figure 1c, 1d) and staining of endothelial cells with isocitrate B4 (red) revealed dense neovascularization (Figure 1c) with incorporation of X-Gal–positive EPCs (arrowheads). High-power magnification (Figure 1d) showed incorporation of EPCs into neovascularization (Figure 1d, arrow) as well as EPCs closely adjacent to, but distinct from, ECs (Figure 1d, arrowhead).
**Systemic Secretoneurin Injection Increases Numbers of Circulating EPCs and Stem Cells**

Secretoneurin was injected intraperitoneally into mice at a dose of 10 μg/d for 7 days. Afterward, PBMCs were subjected to EPC culture (peripheral blood) or FACS analysis (bone marrow cells, peripheral blood).

**EPC Culture**

Cells were stained by acLDL/DiI uptake (red fluorescence) and for binding of FITC BS-1 lectin. Figure 2a demonstrates increase of double-positive spindlelike cells in secretoneurin-treated animals (secretoneurin, 20–100 cells per HPF; solvent, 2.5±1.2 cells per HPF; P<0.01; n=4; Figure 2b).

**FACS Analysis**

Secretoneurin injection significantly increased bone marrow cells positive for c-kit and Sca-1 (control, 1.13±0.36%; secretoneurin, 2.65±0.07%; P<0.05; n=3; Figure 2c). Additionally, numbers of Sca-1-positive cells in the peripheral blood were increased, suggesting mobilization of stem cells by secretoneurin (control, 8.6±4.5%; secretoneurin, 28%±1.8%; P<0.05; n=3; Figure 2d).

**In Vitro Experiments**

**Characterization of Human EPCs Cultured From PBMCs**

EPC formed hemangioblastic islets as described in previous studies. In particular, spindle-shaped cells in the periphery of these islets were double positive for Ulex lectin staining and AcLDL-DiI uptake (Figure 3a). Additionally, EPCs expressed mRNA of the EC-specific markers VEGF receptor 2 (KDR) and CD-31 (Figure 3b).

**Secretoneurin Induces EPC Migration and Inhibits EPC Apoptosis**

Secretoneurin dose dependently activated migration of EPCs, with a maximum effect at a concentration of 10 ng/mL (relative chemotactic index: secretoneurin, 2.02±0.15; P<0.01; n=4; Figure 4a). This effect was comparable to that of VEGF, which is known to induce EPC migration. The migratory effect of secretoneurin on EPCs appears to be specific because it could be blocked with an anti-secretoneurin antibody (Figure 4a).

Compared with cells treated with control buffer, secretoneurin at a concentration of 10 ng/mL reduced cells positive for Annexin-V (green fluorescent, FITC labeled), consistent with decreased EPC apoptosis (Figure 4b).

**Secretoneurin Increases EPC Numbers in Cell Culture**

For quantification, double labeling of EPCs for acLDL/DiI uptake and lectin staining was performed as described previously. Secretoneurin added to human EPC culture dose-dependently increased numbers of EPCs per HPF after 4 days in culture (relative to control): secretoneurin 1 μg/mL, 0.8±0.07; secretoneurin 10 μg/mL, 1.2±0.08; secretoneurin 100 pg/mL, 2.0±0.4; secretoneurin 1 ng/mL, 3.2±0.5; secretoneurin 10 ng/mL, 3.0±0.5; secretoneurin 100 ng/mL, 2.3±0.5; n=3 to 7). Representative images and statistics are shown in Figure 5. Effects were comparable to effects of 50 ng/mL VEGF (3.9±0.7; P<0.01 versus control; n=5), used as positive control. Coincubation of secretoneurin 1 ng/mL with an anti-secretoneurin antibody completely abrogated the secretoneurin-mediated increase in cell number, demonstrating the specificity of secretoneurin-induced effects (P<0.05, secretoneurin 1 ng/mL versus secretoneurin 1 ng/mL plus secretoneurin antibody; Figure 5b).

**Secretoneurin Activates Intracellular MAPK System and Akt Pathway**

Secretoneurin stimulated activation of extracellular signal-regulated kinase (ERK) after 10 minutes and of Akt at 1 ng/mL after 5 minutes. However, activation of ERK seems to
be more prominent than that of Akt (Figure 6a). Coincubation with the MAPK inhibitor PD98059 and the PI3-kinase inhibitor wortmannin inhibited secretoneurin-induced Akt and ERK stimulation (Figure 6b).

To functionally confirm these findings, we preincubated EPCs with PD98059 and wortmannin and performed EPC migration (Figure 6c) and EPC assay for cell number (Figure 6d). As shown before, secretoneurin at a concentration of 10

Figure 2. Systemic application of secretoneurin induces mobilization of EPCs and stem cells to the peripheral circulation. a, b, Secretoneurin (SN) (10 μg/d IP) was injected into mice for 7 days. Afterward, PBMCs were subjected to EPC culture for 4 days and stained for AcLDL/Dii and BS-1 lectin (a). Secretoneurin injection significantly increased numbers of EPCs (b) (*P<0.01, cells per HPF solvent vs secretoneurin; n=4). c, d, In some mice, numbers of c-kit/Sca-1-positive (pos) cells in bone marrow (c) and Sca-1-positive cells in the peripheral circulation (d) were determined by FACS analysis. Secretoneurin increased numbers of stem cells in bone marrow and the peripheral circulation. Ctr indicates control.

Figure 3. Cultured EPCs exhibit EC characteristics. a, PBMCs were subjected to EPC culture for 4 days. Afterward, cells were stained by acLDL/Dii and Ulex lectin. Typically, peripheral cells of hemangioblastic islets were double positive for these markers. b, Additionally, EPCs showed mRNA expression of endothelial markers CD-31 and KDR by RT-PCR.
ng/mL stimulated migration of EPCs and increased cell numbers in the EPC assay. Inhibition of the MAPK pathway reduced secretoneurin-induced cell number and migration, whereas inhibition of the PI3-kinase/Akt pathway only inhibited secretoneurin-induced EPC migration.

Discussion

Recently, cytokines from neuronal origin such as nerve growth factor or several members of the group of neuropeptides such as neuropeptide Y or secretoneurin were reported to induce angiogenesis in addition to their effects on the nervous system. Furthermore, a classic endothelial mitogen, VEGF, was shown to guide vascular supply of the nerves in the skin of embryos, to exert neurotrophic activity, and to improve nerve function in ischemic and diabetic neuropathy. This close interaction between the vascular and the nervous system is not surprising because neuronal cells are very susceptible to hypoxia, and angiogenesis might be an effective mechanism to protect nerve cells from this damage.

Growth of new blood vessels is not restricted to angiogenesis in postnatal life but also occurs via mobilization and incorporation of bone marrow–derived progenitor cells to sites of angiogenesis. Indeed, induction of postnatal vasculogenesis is a well-known feature of angiogenic cytokines such as VEGF. In a recent study, we demonstrated incorporation of EPCs into vessels during reendothelialization of denuded carotid arteries; these cells were positive for Tie2 and an endothelial marker (isolectin B4) and clearly were lining the vessel lumen; thus, these cells represent bone marrow–derived ECs. However, Tie2-positive cells were also reported lying adjacent to, but distinct from, ECs. In the present study, we report that the angiogenic neuropeptide secretoneurin also induces postnatal vasculogenesis in the cornea neovascularization model. Cross sections of corneas showed EPCs integrated into vessels but also cells adjacent to, but distinct from, capillaries. With regard to these differences, at present we can only speculate that Tie2 expressing bone marrow–derived cells might represent several distinct cell types, ie, ECs and a cell type termed Tie2-expressing mononuclear cell. Nevertheless, experimental models inhibiting these cells demonstrated inhibition of blood vessel growth, underscoring the importance of bone marrow progenitors and vasculogenesis in neovascularization.

In corneal neovascularization, secretoneurin-induced angiogenesis seems to be predominant compared with vasculogenesis because more cells are positive for isolectin B4 than for X-Gal (Figure 1c, 1d). However, it remains to be determined whether secretoneurin-induced vasculogenesis is

Figure 4. Secretoneurin (SN) induces EPC migration and inhibits EPC apoptosis. a, Secretoneurin induced directed migration of EPCs in a dose-dependent manner (maximum effect at 10 ng/mL). The effect was comparable to that of VEGF (10 ng/mL) (*P<0.01, relative chemotactic index vs control; n=4). Secretoneurin-induced EPC migration could be blocked by an anti-secretoneurin antibody (Ab) (P<0.01, SN 10 ng/mL vs SN 10 ng/mL + SN Ab; n=3). b, Apoptosis assay of serum-starved EPCs showed decreased FITC-labeled Annexin-V staining (green fluorescence, as a marker of early apoptosis) in secretoneurin-treated cells compared with control cells.
increased in other models of angiogenesis, such as in rapidly growing tumors.

Similar to angiogenic cytokines such as VEGF, secretoneurin is obviously able to mobilize stem cells and EPCs to the peripheral circulation. For VEGF-induced mobilization of stem cells, recently a model of increased release of c-kit by upregulation of metalloproteinases was reported. The mechanism by which secretoneurin mobilizes stem cells from bone marrow remains to be determined.

Stimulation of EPC migration, increase of cell number in vitro, and inhibition of apoptosis are well-known functions of VEGF and drugs such as statins. In the case of statins
particular, activation of intracellular signal transduction pathways was confined to Akt, whereas MAPK was not involved.13,15,16 Secretoneurin induced similar effects on EPCs; however, in contrast to stromal stimulation, the MAPK system seems to play the dominant role, whereas Akt might mediate EPC migration induced by secretoneurin. Secretoneurin was detected in human serum at concentrations of 22 fmol/mL, corresponding to approximately 80 pg/mL.32 Whether this low concentration is able to induce mobilization of EPCs remains to be determined; in our in vitro experiments, secretoneurin was able to increase EPC numbers at concentrations of 100 pg/mL, and therefore it is conceivable that secretoneurin mediates the homeostasis of low circulating EPC levels because other factors reported to induce EPC mobilization (such as VEGF) are usually present at significantly lower concentrations. Furthermore, serum levels of secretoneurin are high immediately after birth (approximately 5-fold compared with adults) and decrease within the first months of life.52 During this time, EPCs have been reported to be present in high circulating levels,33 and secretoneurin might play a role in mobilizing these cells.

Secretoneurin-induced vasculogenesis might also play a role in ischemic conditions because systemic secretoneurin levels of patients with critical limb ischemia were significantly increased compared with controls (controls, 31.9 fmol secretoneurin per milliliter, n = 31; patients, 43.3 fmol secretoneurin per milliliter, n = 27; P < 0.01; R. Kirchmair, MD, and M. Brodmann, MD, unpublished data, 2004).

In conclusion, we demonstrate that the angiogenic neuropeptide secretoneurin induces postnatal vasculogenesis mediated by EPCs. Secretoneurin plays important roles in mobilization, migration, and incorporation of these cells in newly formed vessels.

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