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PRECLINICAL STUDIES

Endothelial Progenitor Cells Participate in Nicotine-Mediated Angiogenesis

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OBJECTIVES We aimed to determine the role of endothelial progenitor cells (EPCs) in cholinergic angiogenesis.

BACKGROUND Recently, we provided evidence for a new angiogenic pathway mediated by endothelial nicotinic acetylcholine receptors (nAChR). Increasing evidence suggests that circulating EPCs also contribute to postnatal neovascularization by homing to sites of neovascularization, a process termed postnatal vasculogenesis. Therefore, we investigated whether nAChR activation increases mobilization and/or recruitment of EPCs to a site of angiogenesis.

METHODS To identify EPCs from reservoirs both inside and outside of the bone marrow and to avoid the adverse effects of total body irradiation, we employed a murine parabiosis model with tie-2-LacZ FvB/N mice connected to wild-type FvB/N mice and induced unilateral hind limb ischemia in the wild-type animal.

RESULTS Administration of nicotine increased capillary density in the ischemic hind limb, and increased soluble Kit ligand plasma levels. The effect of systemic administration was greater than that of local delivery of nicotine (45% vs. 76% increase in capillary density by comparison to vehicle control, intramuscular vs. oral administration of nicotine; $p < 0.05$). Ischemia-induced incorporation of EPC in the control group was rare, but was increased 5-fold by systemic administration of nicotine. Exposure to nicotine in vitro increased EPC count and EPC transmigration. Finally, systemic administration of nicotine increased EPC number in the bone marrow and spleen during hind limb ischemia.

CONCLUSIONS Nicotine treatment increased the number of EPCs in the bone marrow and spleen, and increased their incorporation into the vasculature of ischemic tissue. Administration of nicotine increased markers of EPC mobilization. This study indicates that the known angiogenic effect of nicotine may be mediated in part by mobilization of precursor cells. (J Am Coll Cardiol 2006;48:2553–60) © 2006 by the American College of Cardiology Foundation

Stimulation of endothelial nicotinic acetylcholine receptors (nAChR) induces angiogenesis (1). The endothelial nAChRs are activated by endogenous acetylcholine, as well as by exogenous nicotine. They may play a role in tobacco-related diseases such as tumors, atherosclerosis, and age-related maculopathy, because each of these disorders is characterized by pathological angiogenesis (2). Angiogenesis involves the migration and proliferation of pre-existing, fully differentiated endothelial cells (3). In addition, circulating endothelial progenitor cells (EPCs) may home to sites of neovascularization and differentiate into endothelial cells in situ (4–9). Mobilization of EPCs augments neovascularization of ischemic tissue (5,7,10–12) and may be clinically relevant in the setting of tissue ischemia (2,12,13) or tumor angiogenesis (14–16).

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In this regard, we were struck by our earlier observation that *systemic*, as opposed to *local*, administration of nicotine is more effective in stimulating pathological neovascularization. Specifically, we found that inflammatory angiogenesis was significantly greater when mice received nicotine orally, in comparison to local administration (1). Accordingly, we postulated that nicotine may enhance angiogenesis, in part through, mobilization of EPCs.

METHODS

Animal experiments. MOUSE PARABIOSIS. Parabiotic mouse pairs were created to investigate the mobilization and incorporation into vessels of circulating precursors. Parabiotic partners shared all major histocompatibility antigens and, thus, were free of an immunologic barrier to cell migration and angiogenesis. Unambiguous cell tracking between the mice is possible by assaying for genetic markers unique to one animal in the pair. In brief, we surgically joined 10-week-old male mice carrying a

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Abbreviations and Acronyms

DMXB = 3-(2,4)-dimethoxybenzylidene anabaseine
EPC = endothelial progenitor cell
FITC = fluorescein isothiocyanate
HUVEC = human umbilical vein endothelial cell
nAChR = nicotinic acetylcholine receptor
SDF = stem cell-derived factor
VEGF = vascular endothelial growth factor

β-galactosidase reporter gene under the control of the murine Tek (Tie2) promoter ($LacZ^+$; FVB/N-TgN(Tie2LacZ)182Sato; Jackson Laboratory, Bar Harbor, Maine) with female age- and strain-matched FvB/N wild-type mice ($LacZ^-$) (17). Mice were anesthetized with xylazine and ketamine HCl (1.67 mg per 10 g of body weight intraperitoneally). A lateral surface of each mouse was shaved, the skin was incised from the olecranon to the knee joint of each mouse, and the subcutaneous fascia was bluntly dissected to create about 1/2 cm of free skin. The mice were joined at the olecranon and knee joints by a single 2-0 silk suture and tie, and the dorsal and ventral skin flaps were approximated by staples (17–20). Peripheral blood chimerism of parabiotic mice was determined by CD45 allotype analysis (21). Parabiotic mice, in which partners differed at the CD45 locus (CD45.1 and CD45.2), showed cross circulation as early as 3 days after surgical joining, and blood chimerism reached 50% by days 7 to 10. Thus, we estimate that about 50% of circulating EPCs theoretically would be derived from the transgenic animal.

MURINE MODEL OF HIND LIMB ISCHEMIA. Hind limb ischemia was surgically induced (22). Briefly, the proximal portion of the femoral artery including the superficial and the deep branch as well as the distal portion of the saphenous artery were occluded. For parabiotic pairs, the procedure was performed on day 30, well after the anastomosis had healed and cross circulation had been stably attained, in the female $LacZ^-$ mouse only. Subsequently, animals were randomized to local intramuscular injections (of vehicle or nicotine 0.03 μg/kg body weight, directly into the ischemic hind limb; Sigma-Aldrich, St. Louis, Missouri), or to systemic oral administration (of vehicle or nicotine 100 μg/ml drinking water with 2% saccharine) (1). As reported earlier, the maximum angiogenic effect for induced by local intramuscular administration of nicotine is observed at 0.03 μg/kg body weight (1). The dose of nicotine administered by the oral route was calculated to deliver a similar average tissue level of nicotine. Of note, each parabiotic pair of animals was administered the same treatment (e.g., oral nicotine solution or vehicle). Serum cotinine levels were measured by enzyme-linked immunoadsorbent assay (STC Technologies, Tucson, Arizona). All animal experiments were approved by the Administrative Panel on Laboratory Animal Care (A-PLAC) at Stanford University School of Medicine.

HISTOLOGIC ANALYSIS. Three weeks after induction of hind limb ischemia, limb muscles were harvested and sectioned. Cells in the ischemic and nonischemic hind limbs of the female $LacZ^-$ mice that derived from their male partners were identified by monoclonal antibodies against β-galactosidase (Sigma). Sections were double-stained with fluorescent antibodies against β-galactosidase and antibodies against the endothelium-associated antigens CD31 (BD Bioscience, San Jose, California). Progenitor cell frequency was defined as the number of vessels containing transgenic endothelial cells divided by the total vessels examined in representative sections.

ASSESSMENT OF EPC MOBILIZATION BY FLOW CYTOMETRY. Bone marrow cells were harvested by flushing tibias and femurs of donor mice and filtered (70 μm). Spleens were mechanically minced using syringe plungers and laid over Ficoll to isolate mononuclear cells (splenocytes). C57BL/6J mice were randomized to vehicle, nicotine (100 μg/ml drinking water), granulocyte-macrophage colony-stimulating factor (GM-CSF) (25 μg/kg for 3 consecutive days each; PeproTech, Rocky Hill, New Jersey) in the presence or absence of ischemia. At each time point (baseline, 3, 7, 14 days), peripheral blood was obtained from the inferior vena cava and the right ventricle. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated antibodies against mouse CD34 and phycoerythrin (PE)-conjugated antibodies against Flk-1 (BD Bioscience) and analyzed by FACS-Vantage flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey). We analyzed the lymphocyte gate instead of using a third surface marker such as CD45. Therefore, we cannot exclude that a subset of the "EPCs" are $CD45^+$. Staining was performed in the presence of saturating concentrations of rat monoclonal unconjugated antibodies against Fc receptors (anti-CD16/32, BD Bioscience) to reduce nonspecific binding. Isotype-identical antibodies served as controls (IgG_1-PE and IgG_{2a}-FITC; BD Bioscience). Each analysis included 100,000 events. Data were analyzed using FloJo software (Becton Dickinson).

PLASMA MEASUREMENT OF S-KIT LIGAND. We used a commercially available ELISA kit (R&D Systems, Minneapolis, Minnesota) to measure plasma levels of soluble kit ligand after 6 weeks of administration of vehicle or nicotine (ad libitum at a concentration of 100 ug/ml with 0.4% saccharine).

In vitro cell culture experiments. EPC CULTURE ASSAY. Mononuclear cells were isolated from 10-week-old C57BL/6J mice by density-gradient centrifugation with Biocoll from peripheral blood and spleen homogenates (these animals were not treated with nicotine). Immediately after isolation, 5×10^6 mononuclear cells were plated on 24-well culture dishes coated with human fibronectin

(Sigma) and maintained in endothelial basal medium (EBM, Clonetics Corp., San Diego, California) supplemented with 20% fetal calf serum. Treatment with increasing concentrations of nicotine ranging from 0.01 μmol/l (10^{-8} mol/l) to 10.0 μmol/l (10^{-5} mol/l) was initiated after 48 h of culturing the mononuclear cells. As a marker of endothelial phenotype, we assessed cellular uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (DilacLDL; 2.4 μg/ml) after incubation at 37°C for 1 h. Cells were then fixed with 2% paraformaldehyde for 10 min, and lectin staining was performed by incubation with fluorescein isothiocyanate FITC-labeled Ulex europaeus agglutinin I (lectin, 10 μg/ml; Sigma) for 1 h. Dual-stained cells were judged to be derived from EPCs and were counted in 5 randomly selected fields.

TRANSMIGRATION ASSAY. Human umbilical vein endothelial cells (HUVEC) (1×10^5 cells/well; up to second passage; BioWhittaker, Walkersville, Maryland) were plated on polycarbonate membrane (3-μm pore filters; Corning Costar, Acton, Massachusetts) coated with collagen I (10 μg/ml; Becton Dickinson) (8) to obtain confluent endothelial monolayers. Confluency was confirmed by measuring permeability for FITC-dextran 3,000 (Molecular Probes, Eugene, Oregon). Monolayers of endothelial cells were pre-treated for 4 h with nicotine (0.1 μmol/l) or vehicle. In parallel, EPCs had been cultured using the methods described in the previous text. Immediately before the transmigration assay, cultured EPCs were detached using 1 mmol/l ethylenediaminetetraacetic acid in phosphate-buffered saline, resuspended in 500 μl of endothelial basal medium, labeled with CellTracker (Molecular Probes), counted, and 10^5 cells were placed in the upper chamber on top of the HUVEC monolayer. The chamber was placed in a 24-well culture dish containing nicotine (1.0 nmol/l to 1.0 μmol/l) or human recombinant stem cell-derived factor (SDF)-1 (100 ng/ml), respectively. After 24 h of incubation at 37°C, the lower side of the filter was washed with phosphate-buffered saline and fixed with 2% paraformaldehyde. Fluorescently labeled EPCs migrating into the lower chamber were counted manually in 5 random microscopic fields.

STATISTICAL ANALYSIS. Values are expressed as mean ± SD. Comparisons between groups were analyzed by *t* test (2-sided) or analysis of variance for experiments with more than 2 subgroups. Post hoc range tests and pair wise multiple comparisons were performed with the *t* test (2-sided) with Bonferroni adjustment. Comparison of categorical variables was generated by the Pearson chi-square test. All analyses were performed with SPSS 10.0 (SPSS Inc., Chicago, Illinois). The p values <0.05 were considered statistically significant.

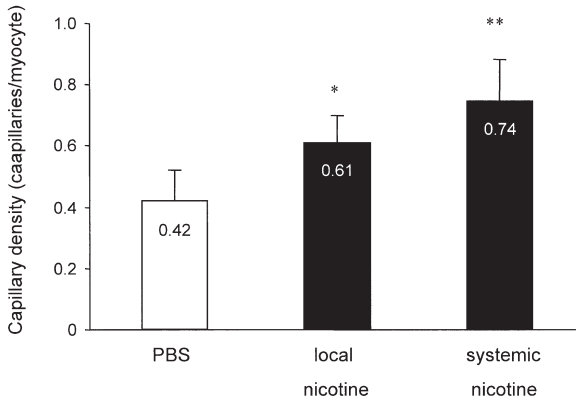

Figure 1. Nicotine increases capillary density in the ischemic hind limb. Murine model of hind limb ischemia: nicotine stimulates angiogenesis as assessed by an increase in capillary density. Systemic treatment with nicotine resulted in a significantly higher angiogenic response as compared with local administration of nicotine into the ischemic hind limb (n = 7 each group). PBS = phosphate-buffered saline. *$p < 0.01$ versus control; **$p < 0.05$ versus local nicotine.

RESULTS

Systemic administration of nicotine induces greater angiogenesis. As before, we observed that nicotine enhanced angiogenic response to ischemia. However, systemic was more effective than local administration of nicotine. Intramuscular administration of nicotine induced a 46% increase in neovascularization as compared with phosphate-buffered saline (capillary/myocyte ratio: control group 0.42 ± 0.10 vs. local nicotine 0.61 ± 0.09; $p < 0.01$; n = 7 each group) (Fig. 1). Local injection of nicotine did not lead to detectable cotinine levels in the peripheral blood. Systemic treatment with nicotine via the drinking water (100 μg/ml drinking water) increased cotinine levels to 246 ± 61 ng/ml and increased capillary density to a greater degree (capillary/myocyte ratio 0.74 ± 0.14 vs. 0.61 ± 0.09; $p < 0.05$ systemic vs. local nicotine). On day 7 after unilateral ligation of the femoral artery, the group exposed to systemic administration of nicotine had significantly greater plasma levels of vascular endothelial growth factor (VEGF) (control: 33.8 ± 7.5 pg/ml, local nicotine: 48.5 ± 9.6 pg/ml, systemic nicotine: 86.0 ± 15.5 pg/ml; $p < 0.01$).

Nicotine increases the number of EPCs in vivo. We hypothesized that the greater effect of nicotine when given systemically was due to recruitment of EPCs. To address this issue, we determined if systemic administration of nicotine could increase EPCs in the setting of hind limb ischemia. Analysis of different cell compartments by flow cytometry revealed that, in the absence of ischemia, CD34/Flk-1-positive cells comprised only 0.24 ± 0.14% of the cells in the bone marrow (Figs. 2A and 2B), 1.18 ± 0.21% of the cells in the spleen, and only 0.03 ± 0.02% of the cells in the peripheral blood. GM-CSF treatment for 3 days significantly increased the number of CD34/Flk-1-positive cells in all 3 compartments, whereas nicotine treatment without hind limb ischemia did not result in a significant increase of CD34/Flk-1-positive cells (data not shown).

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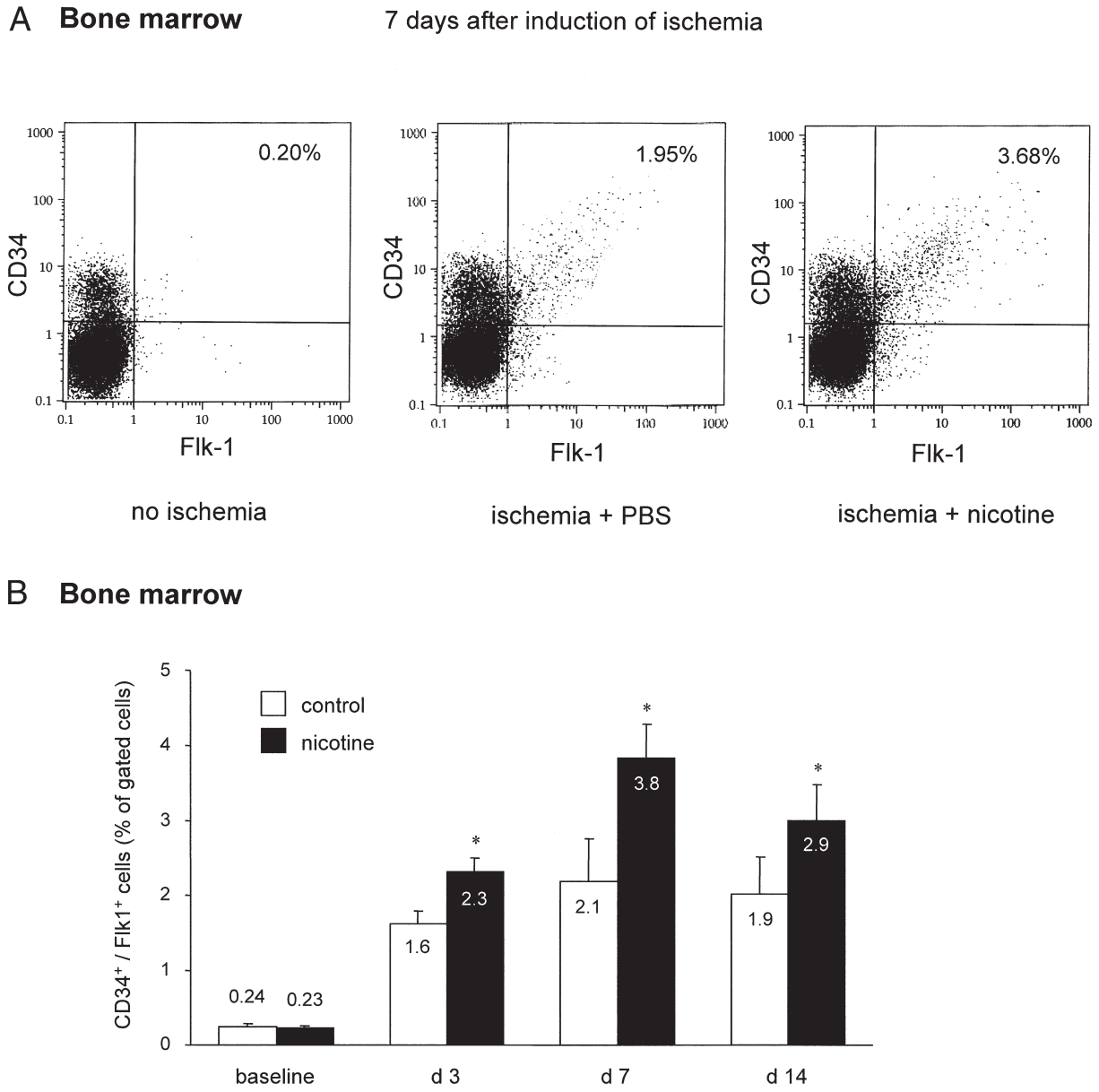

Figure 2. Quantitation of endothelial progenitor cells by flow cytometry. Frequency of $CD34^+$ and Flk-1^+ cells in control animals without ischemia, with ischemia, and with ischemia and nicotine treatment **(A)**. Histogram showing number of endothelial progenitor cells in the bone marrow at increasing intervals after induction of ischemia and initiation of oral nicotine **(B)**. For each group, n = 5. *p < 0.01 versus control for each respective time point.

After unilateral ligation of the common femoral artery, the number of $CD34^+$/Flk-1^+ cells in the bone marrow increased in control animals. The number of $CD34^+$/Flk-1^+ cells in the bone marrow was further increased by systemic administration of nicotine, with a maximum effect observed at day 7, and a persistent effect up to day 14 (Figs. 2A and 2B). We also observed an increase in the number of $CD34^+$/Flk-1^+ cells in the spleen on day 3 (+23%; control group 3.65 ± 0.53% $CD34^+$/Flk-1^+ cells vs. nicotine 4.47 ± 0.48%; $p < 0.05$; n = 5 each group) as compared with vehicle-treated animals ($p < 0.05$). Results for day 7 were +22%, and for day 14 an increase of +21% was observed.

Mobilization of stem and progenitor cells is dependent on local secretion of matrix metalloproteinase in the bone marrow, which results in the subsequent release of soluble Kit ligand (also know as stem cells factor) (23,24). Therefore, levels of soluble Kit ligand have been used as a surrogate marker for stem cell mobilization. Plasma levels of s-kit ligand were increased 6-fold in animals receiving oral nicotine (87.9 ± 27.2 pg/ml vs. 14.4 ± 2.9 pg/ml; n = 5 in each group; $p < 0.005$).

Nicotine stimulates EPC incorporation in vivo. To confirm that nicotine increased the mobilization of EPCs, and to determine the functional significance of increased EPC mobilization, the following experiment was performed. To track incorporated EPCs in the ischemic and nonischemic tissue, we used a murine model of parabiosis. Hind limb ischemia was induced 30 days after transgenic mice (tie2-

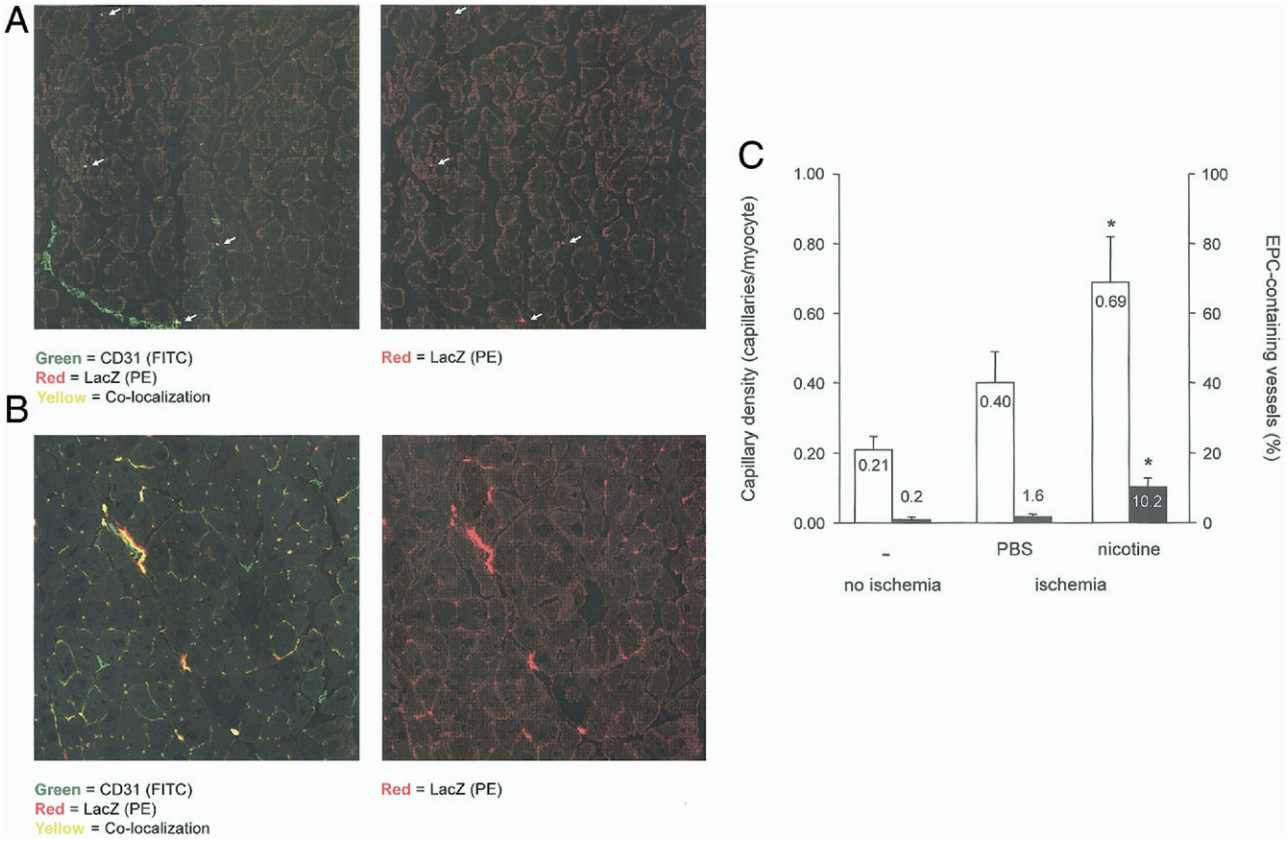

Figure 3. Incorporation of mobilized endothelial progenitor cells (EPCs). Representative cross sections of ischemic hind limbs of female animals treated with vehicle **(A)** or nicotine **(B)**, respectively. All endothelial cells stain for CD31 (fluorescein-isothiocyanate-labeled; **green**). Those endothelial cells that are derived from the male transgenic animal also express β-galactosidase (**red fluorescent** phycoerythrin [PE]-labeled secondary antibody) resulting in a double stain that is **yellow**. Histogram showing mean values for capillary density and percentage of EPC-containing vessels (from an analysis of 10 randomly selected high-power fields in each group) **(C)**. Nicotine administration increased capillary density as well as the number of vessels containing endothelial cells that had been derived from EPC. For each group, n = 5. **Open bars** = capillary density ($CD31^+$); **solid bars** = EPC-containing vessels. *$p < 0.01$ versus phosphate-buffered saline (PBS).

LacZ) were surgically connected to wild-type animals. In vehicle-treated pairs, capillary density increased after induction of ischemia, but incorporation of EPCs in the ischemic tissue was infrequent. Only 1.6 ± 1.0% of the vessels in the ischemic tissue co-localized for LacZ and CD31 (Figs. 3A and 3C). Similar results were obtained for co-localization for LacZ and Flk-1 (1.4 ± 0.5%). In animals systemically treated with nicotine, capillary density was increased by about 6-fold (Figs. 3B and 3C). It seems likely that only 50% of the EPCs are derived from the transgenic animal. Accordingly, one might estimate that 21.0% of the vessels in nicotine-treated animals incorporated EPC. However, in Figure 3C, we only show the percentage of vessels containing EPCs that could be observed by B-gal staining (i.e., just those vessels containing EPCs derived from the male animal).

Nicotine increases EPC number in colony formation assay. To further delineate the mechanisms underlying the increase in EPC number in vivo, we evaluated the direct in vitro effect of nicotine on EPC colony formation. After culturing mononuclear cells as described above for 48 h, cells were stimulated with nicotine for an additional 48 h. After washing with medium, adhering cells were stained with DilacLDL. We observed a dose-dependent and bimodal effect on the number of DilacLDL-positive cells. A significant increase in DilacLDL-positive cells was observed for 0.1 μmol/l and 1.0 μmol/l nicotine, whereas 10.0 μmol/l of nicotine resulted in a reduced number (Figs. 4A to 4C).

Nicotine stimulates EPC transmigration. To further delineate the mechanisms underlying the increase in EPC mobilization in vivo, we studied EPC transmigration through an endothelial monolayer using a modified Boyden chamber assay. Pre-treatment of the EPC with nicotine increased the number of transmigrated EPC by 2-fold (Figs. 5A to 5C). Pre-treatment of the HUVEC monolayer with nicotine appeared to have an additive effect. The potent migragen SDF-1 increased transmigration, with an additive effect of nicotine.

DISCUSSION

The salient findings of our study are that systemic exposure to nicotine augments the number of EPCs in the bone marrow and spleen and that this increase is associated with a marked increase in angiogenesis in ischemic tissue. The

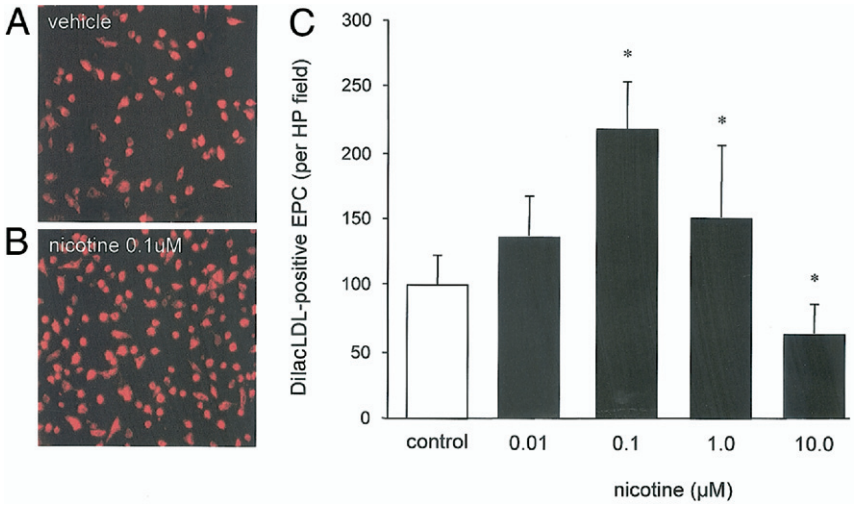

Figure 4. Nicotine pre-treatment increases endothelial progenitor cell (EPC) number in vitro. 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (DilacLDL) uptake **(A and B)** of isolated mononuclear cells was determined by fluorescence microscopy. Treatment of the cells with nicotine for 48 h increased the number of adherent DilacLDL+ cells per high-power (HP) field in a dose-dependent fashion **(C)**. For each group, n = 5. *p < 0.01 versus control.

nicotine-induced enhancement of angiogenesis is associated with greater numbers of EPCs incorporating into the ischemic hind limb. We also observed that systemic exposure to nicotine increased s-kit ligand, a plasma marker of stem and progenitor cell mobilization. Finally, in vitro studies indicated that nicotine could increase transmigration of EPCs.

We have previously described an endothelial nAChR that mediates angiogenesis (25). Like neuronal nAChRs, the endothelial nAChR is a pentameric protein that forms a ligand-gated calcium channel, normally activated by endogenous acetylcholine (26). Nicotine may also activate this receptor to induce angiogenesis. There are a wide variety of neuronal and extraneuronal nAChRs, each composed of 5 subunits (i.e., α_{1-10}, β_{1-4}, γ, Δ, and ϵ (26). In the endothelial cell, the predominant nAChR is an α_7 homomer, the expression of which increases with hypoxia (25). The endothelial expression of the nAChR is rather low under basal conditions, but is up-regulated by hypoxia (25). This regulated expression of the endothelial nAChR may explain why nicotine did not mobilize EPCs in the absence of ischemia (present report), or enhance angiogenesis in normal tissue (25,27). It is possible that a permissive factor released from the ischemic tissue renders the bone marrow responsive to nicotine. Local hypoxia is known to increase systemic levels of angiogenic cytokines, including VEGF.

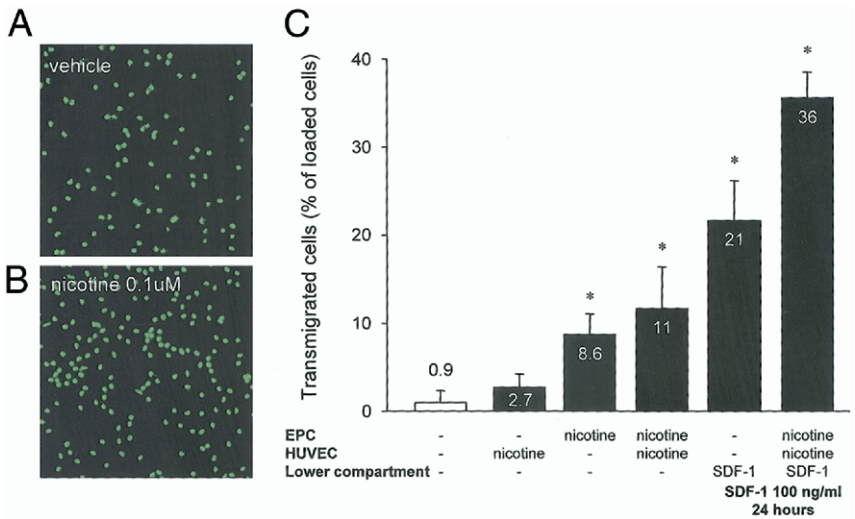

Figure 5. Nicotine stimulates endothelial progenitor cell (EPC) transmigration. Nicotine stimulates EPC transmigration through human umbilical vein endothelial cell (HUVEC) monolayer: transmigrated cells at the bottom of the porous membrane in the vehicle **(A)** and nicotine **(B)** group. Endothelial progenitor cells were labeled with CellTracker before co-incubation. Significantly more transmigrated EPCs were observed when EPCs were pre-treated with nicotine **(C)**. Pre-treatment of the endothelial cell monolayer with nicotine also increased EPC transmigration, and the effects of nicotine treatment on EPCs or HUVECs appeared to be additive with each other, or with stem-cell-derived factor (SDF)-1 **(C)**. For each group, n = 5. *p < 0.01 versus control.

Unpublished work from our laboratory indicates the VEGF stimulates the expression of several endothelial nAChR subunits. Further evidence of a role for VEGF in nicotine-mediated angiogenesis comes from our published observations that the endothelial tube formation induced by nicotine or 3-(2,4)-dimethoxybenzylidene anabaseine is antagonized by VEGF-neutralizing antibodies (25).

We have previously shown that stimulation of the endothelial nAChR induces endothelial cell proliferation, migration, and tube formation in vitro (1,25). We have provided evidence that nicotine promotes tumor angiogenesis and tumor growth (1). Second-hand tobacco smoke also promotes tumor angiogenesis and tumor growth, an effect that is abolished by the nAChR antagonist mecamylamine (28). Second-hand tobacco smoke increased serum VEGF concentrations and circulating levels of EPCs (as documented by flow cytometry analysis). These effects of second-hand tobacco smoke were inhibited by mecamylamine (28), indicating that second-hand tobacco smoke-induced recruitment of EPCs is mediated by the nAChR. The nicotine-induced mobilization of EPCs, and their contribution to tumor angiogenesis, has also been demonstrated by Natori et al. (29). However, it must be noted that in the current manuscript, we have not proven that the effects of nicotine are mediated by the nAChR, nor have we shown the existence of the nAChR on EPCs. Nicotine could exert its effects through receptor-independent mechanisms or through other neuroeffector, chemosensory, or inflammatory mechanisms. Furthermore, we have not proven that the EPCs incorporating into the ischemic region are derived solely from the bone marrow. Sources other than the bone marrow have been postulated for EPCs and other progenitor cells (30–32). Although we have shown that systemic exposure to nicotine recruits circulating cells of endothelial lineage to an area of ischemia, we have not excluded a role for nicotine acting locally to increase EPC incorporation. One might imagine that nicotine could act locally, in the setting of ischemia, to augment the release or effects of other factors that mobilize EPCs, such as VEGF. Indeed, nicotine increases the endothelial expression of VEGF (25,27).

Another form of pathological angiogenesis occurs during the growth of atherosclerotic plaques. Larger atherosclerotic plaques in the coronary arteries are heavily vascularized by expansion of the vasa vasorum (33,34). Administration of antiangiogenic agents to hypercholesterolemic apolipoprotein-E-deficient mice suppresses plaque growth (35). We have shown that administration of nicotine to hypercholesterolemic mice accelerates plaque neovascularization and progression (1).

The mobilization of EPCs contributes to angiogenesis. To determine if the angiogenic effects of nicotine might be mediated, in part, by EPC mobilization, we used a model of mouse parabiosis (17,19). Cells arising from one partner can be differentiated from the other by virtue of stable genetic markers such as gender chromosomes or the presence of a reporter transgene such as LacZ. Our goal was to eliminate biases inherent in models that require pre-selection of a given type or source of the cells, and to avoid manipulations (such as total body irradiation) required to overcome immunologic or physiological barriers between the putative precursor cells and the experimental hosts. Our studies indicate that nicotine enhances EPC mobilization. Alternatively or in addition, nicotine may enhance homing, survival, or even the expression of EPC surface markers.

It seems counterintuitive that nicotine could stimulate therapeutic angiogenesis as in the current model. Nevertheless, we have previously observed in mouse and rabbit models of hind limb ischemia that both angiogenesis and arteriogenesis are enhanced by oral or intramuscular nicotine (1,36). Furthermore, in the diabetic db^{-}/db^{-} mouse, wound healing is accelerated, and wound vascularity increased 2-fold, by topical administration of nicotine, or another nAChR agonist, epibatidine (37). Our observation that nicotine can mobilize EPCs seems to conflict with reports that human smokers have fewer circulating EPCs (38), which also exhibit impaired function (39). Furthermore, tobacco cessation rapidly restores the number of circulating progenitor cells (40). A possible explanation for the paradoxical findings in the current investigation is that by comparison to nicotine, tobacco smoke is composed of 4,000 different compounds. Some of the components of tobacco are known to be cytotoxic or mutagenic, and/or induce oxidative stress. Thus, the rather brief exposure to nicotine in the current study is a qualitatively different stimulus than chronic exposure to tobacco smoke. Indeed, Wang et al. (41) have performed in vitro studies showing that nicotine dose-dependently enhances EPC proliferation, migration, adhesion, and tubule formation.

To conclude, we find that in the setting of ischemia, nicotine mobilizes EPCs, which incorporate into the vasculature of the ischemic tissue. This effect may be due to direct actions of nicotine on EPC proliferation, migration, and/or mobilization, as suggested by in vitro models and plasma markers used in this investigation. These findings indicate the existence of a novel pathway for therapeutic modulation in diseases characterized by pathological or insufficient angiogenesis.

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