Nicotine stimulates angiogenesis and promotes tumor growth and atherosclerosis

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We provide anatomic and functional evidence that nicotine induces angiogenesis. We also show that nicotine accelerates the growth of tumor and atheroma in association with increased neovascularization. Nicotine increased endothelial-cell growth and tube formation *in vitro*, and accelerated fibrovascular growth *in vivo*. In a mouse model of hind-limb ischemia, nicotine increased capillary and collateral growth, and enhanced tissue perfusion. In mouse models of lung cancer and atherosclerosis, we found that nicotine enhanced lesion growth in association with an increase in lesion vascularity. These effects of nicotine were mediated through nicotinic acetylcholine receptors at nicotine concentrations that are pathophysiologically relevant. The endothelial production of nitric oxide, prostacyclin and vascular endothelial growth factor might have a role in these effects.

Angiogenesis is a complex, combinatorial process that is regulated by a balance between pro- and anti-angiogenic molecules[1]. This balance is disturbed in the setting of pathological angiogenesis, which is a feature of tobacco-related diseases (for example, cancer and atherosclerosis). It is not known whether smoking directly influences the pathological angiogenesis that accompanies tobacco-related diseases.

Nicotine is responsible for the psychoactive actions and addictive properties of tobacco[2]. Nicotine acts via nicotinic acetylcholine receptors (nAChR) that mediate fast synaptic transmission. Non-neuronal cells, including endothelial cells, also express nAChR (ref. 3). Villablanca found that nicotine stimulates endothelial-cell proliferation via nAChR (ref. 4). Moreover, studies by Zhang *et al*. showed an increased expression of endothelial nitric-oxide synthase (eNOS) after nicotine stimulation of human endothelial cells in coronary arteries[5]. Other studies have also shown that nicotine can promote the release of basic fibroblast growth factor (bFGF), prostacyclin and endothelin from endothelial cells and smooth-muscle cells (SMCs), respectively[6–10]. Whereas these *in vitro* findings are consistent with a pro-angiogenic effect of nicotine, increased angiogenesis in response to nicotine has not been demonstrated. In fact, there are numerous reports indicating that nicotine, or smoking, causes endothelial injury and might thus impair angiogenesis[9,11–13]. Using a variety of models, we demonstrate for the first time that nicotine stimulates angiogenesis in the settings of inflammation, ischemia, tumor and atherosclerosis. Nicotine promotes the growth of atherosclerotic plaques and tumors at least in part by stimulating pathological angiogenesis.

Nicotine is an agent of angiogenesis

Villablanca has shown that nicotine stimulates the proliferation of bovine pulmonary artery endothelial cells[4]. Building on these findings, we studied the effects of nicotine on endothelial-cell proliferation, apoptosis and morphological changes using early passages of two primary human endothelial-cell lines. Nicotine increased the cell number of cultured human umbilical-vein endothelial cells (HUVECs) and human coronary-artery endothelial cells (HCAECs) by up to three-fold (Fig. 1*a*). The maximal effect occurred at concentrations of nicotine (1×10^{-8} to 1×10^{-7} M) found in the plasma of smokers[14]. Moreover, nicotine reduced the number of apoptotic cells after 24-hour exposure to hypoxia (3% oxygen). Flow cytometry analysis revealed that 7% (95% confidence interval (CI) 3–10) of cells treated with 1×10^{-7} M nicotine were positive for annexin V compared with 13% (95% CI 11–17) of control cells ($P < 0.001$). The anti-apoptotic effect of nicotine was blocked by hexamethonium (1×10^{-4} M), a specific antagonist of nAChR.

Later stages of angiogenesis require morphological alterations of endothelial cells resulting in lumen formation. In growth-factor–free collagen gel, angiogenic factors induce cultured endothelial cells to form a network of capillary-like structures[15]. After we seeded the cells on growth-factor–free collagen gel, the control medium did not induce morphological changes in HUVECs or HCAECs (Fig. 1*b*). However, addition of vascular endothelial growth factor (VEGF; 50 ng/ml) stimulated network formation within 24–48 hours (Fig. 1*c*). Nicotine (1×10^{-8} M) had identical effects on endothelial-cell morphology (Fig. 1*d*), which were completely antagonized by hexamethonium (1×10^{-4} M).

Although these findings are consistent with the alterations in endothelial growth and morphology that occur in angiogenesis, they do not prove that nicotine induces angiogenesis *in vivo*. Accordingly, we implanted a disc angiogenesis system subcutaneously in the flanks of C57BL6J mice. When removed two weeks later, the discs showed a fibrovascular ingrowth (Fig. 2*a*). Fibrovascular growth is accelerated when angiogenic agents are added to the disc matrix[16]. The addition of bFGF (to a pellet in the center of the disc for local drug delivery) increased the fibrovascular ingrowth from 12.2 mm^2 (95% CI 10.1–13.8) to 23.1 mm^2 (95% CI 17.9–25.8) ($P < 0.001$). Similarly, when we added nicotine, the area of fibrovascular growth increased to 20.1 mm^2 (95% CI 18.5–22.6; $P < 0.001$) (Fig. 2*b*). Systemic administration

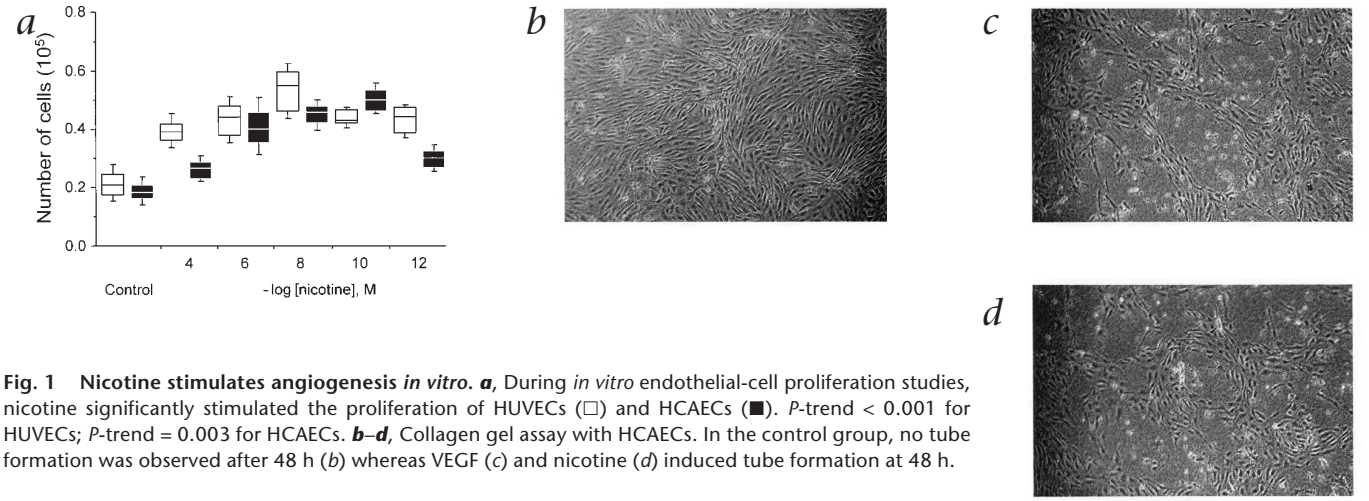

Fig. 1 Nicotine stimulates angiogenesis *in vitro*. *a*, During *in vitro* endothelial-cell proliferation studies, nicotine significantly stimulated the proliferation of HUVECs (□) and HCAECs (■). *P*-trend < 0.001 for HUVECs; *P*-trend = 0.003 for HCAECs. ***b*–*d*,** Collagen gel assay with HCAECs. In the control group, no tube formation was observed after 48 h (*b*) whereas VEGF (*c*) and nicotine (*d*) induced tube formation at 48 h.

of nicotine in the drinking water (100 μg/ml) increased the area of fibrovascular growth to an even greater degree at 38.9 mm^2 (95% CI 29.3–58.6; $P < 0.001$). Both the NOS inhibitor L-nitroarginine and the cyclooxygenase (COX) inhibitor indomethacin blocked the angiogenic effect of nicotine, indicating that synthesis of nitric oxide and prostacyclin is required for the observed effect of nicotine.

To determine whether nicotine enhances angiogenesis in response to ischemia and whether this has functional effects on tissue perfusion, we used a mouse model of hind-limb ischemia[17]. After unilateral ligation of the femoral artery, we administered nicotine or saline by intramuscular injections into the ischemic hind limb on a daily basis for a period of three weeks. In a first dose-escalating study, five groups of mice (each $n = 6$) received 0, 0.003, 0.03, 0.3 or 3.0 μg/kg of nicotine. Nicotine influenced angiogenesis in a bimodal fashion (Fig. 3*a*). At an intermediate nicotine dose of 0.03 μg/kg, nicotine doubled the angiogenic response from 0.35 (95% CI 0.09–0.46) to 0.69 (95% CI 0.45–1.06) capillaries/myocyte ($P < 0.001$). In contrast, at concentrations of 3 μg/kg or greater, angiogenesis was not increased, and we observed local cytotoxicity with evidence of interstitial edema and myocyte necrosis. Neither ipsilateral nor contralateral injections of nicotine showed any effect in the non-ischemic hind limb (data not shown). The co-administration of the nAChR antagonists mecamylamine (1.0 μg/kg) or hexamethonium (1.0 μg/kg) abolished the angiogenic effect of nicotine (Fig. 3*b*). In contrast, the muscarinic acetylcholine receptor antagonist atropine did not significantly reduce the angiogenic response to nicotine.

We achieved similar results when we administered nicotine systemically (100 μg/ml in drinking water). At this dose, plasma nicotine levels in the mice were similar to those observed in moderate smokers. Capillary density in the ischemic hind limb increased from 0.38 (95% CI 0.15–0.49) capillaries/myocyte for the control group to 0.71 (95% CI 0.55–1.01) capillaries/myocyte for the nicotine group ($P < 0.001$), whereas capillary density did not change in the non-ischemic leg.

An increase in capillary density does not necessarily increase local blood flow without a concomitant increase in collateral vessel growth. We investigated this in another group of experimental mice. After unilateral ligation of the femoral artery, we treated the ischemic limb with intramuscular injections of saline, nicotine (0.1 μg/kg) or epibatidine (0.001 μg/kg). Epibatidine is an agonist with a 100-fold greater affinity for the nAChR compared with nicotine[18]. We identified collaterals by double-staining for CD31 (in endothelial cells) and α-actin (in SMCs) using confocal microscopy. We then quantified the number of collaterals on cross-sections by identifying vascular lumen lined by $CD31^+$ endothelia, which were coated by an actin-positive vascular smooth-muscle wall, with vessel diameters of at least 0.02 mm (Fig. 3*c*). Compared with the non-ischemic hind limb, the number of collateral vessels in the ischemic limb of the control mice increased by two-fold (Fig. 3*d*), whereas the median size of the collateral vessels did not change (Fig. 3*e*). In the setting of ischemia, treatment with nicotine or epibatidine increased the number of collateral vessels by about five-fold (Fig. 3*d*) and almost doubled the median diameter of the vessels (Fig. 3*e*).

To determine whether the effects of nicotinic

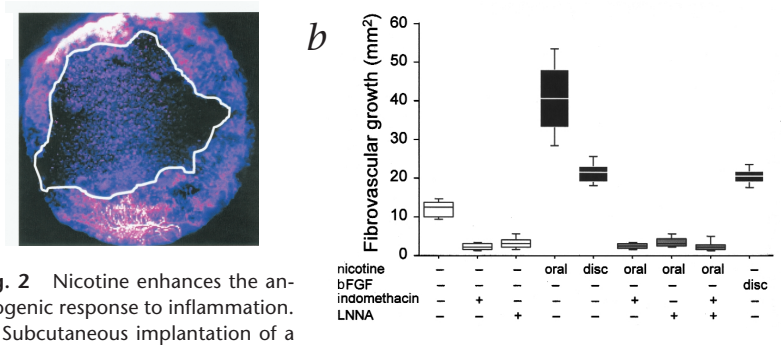

Fig. 2 Nicotine enhances the angiogenic response to inflammation. ***a*,** Subcutaneous implantation of a polyvinyl sponge for 2 wk resulted in ingrowth of capillaries. Perfused vessels were identified by injection of space-filling fluorescent microspheres (border of the neovascularization is outlined). ***b*,** Nicotine, given either locally into the pellet in the center of the disc or systemically in the drinking water significantly increased the area of fibrovascular growth ($P < 0.001$ versus control). Total fibrovascular growth was directly proportional to the vascularized area and was used as an index of angiogenesis. Indomethacin, L-nitroarginine (LNNA), or both reversed this angiogenic effect of nicotine ($P < 0.001$ versus nicotine alone). bFGF served as a positive control.

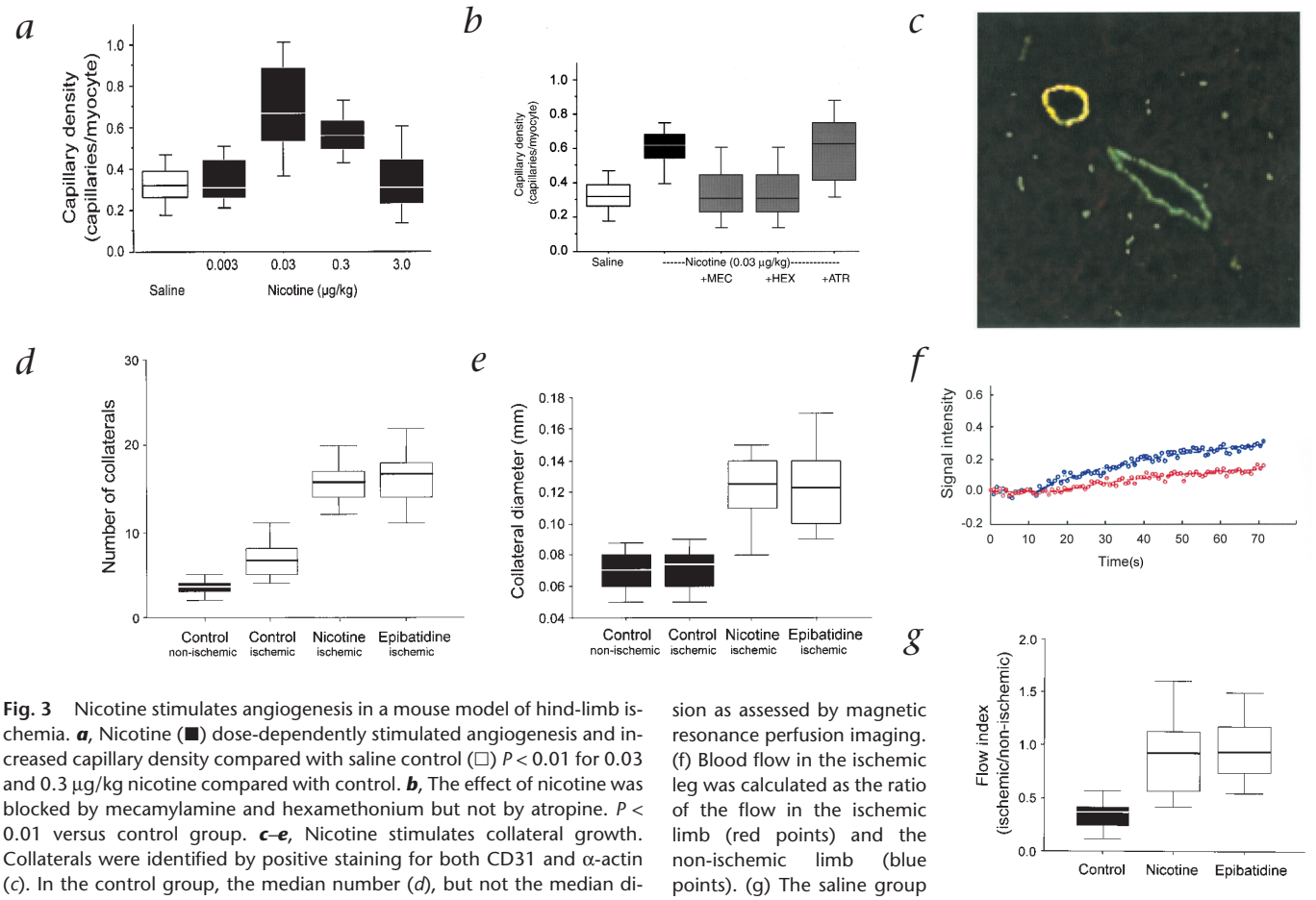

Fig. 3 Nicotine stimulates angiogenesis in a mouse model of hind-limb ischemia. ***a***, Nicotine (■) dose-dependently stimulated angiogenesis and increased capillary density compared with saline control (□) $P < 0.01$ for 0.03 and 0.3 μg/kg nicotine compared with control. ***b***, The effect of nicotine was blocked by mecamylamine and hexamethonium but not by atropine. $P <$ 0.01 versus control group. ***c–e***, Nicotine stimulates collateral growth. Collaterals were identified by positive staining for both CD31 and α-actin (*c*). In the control group, the median number (*d*), but not the median diameter (*e*), of the collateral vessels was increased ($P < 0.01$). In the nicotine and the epibatidine groups, both number and diameter of the collaterals were significantly higher as compared with control ($P < 0.01$ for both). ***f*** and ***g***, Stimulation of the nAChR promotes angiogenesis and tissue perfusion as assessed by magnetic resonance perfusion imaging. (f) Blood flow in the ischemic leg was calculated as the ratio of the flow in the ischemic limb (red points) and the non-ischemic limb (blue points). (g) The saline group had impaired blood flow in the ischemic limb whereas the nicotine group and the epibatidine group, respectively, showed significantly enhanced blood flow in the ischemic limb ($P < 0.01$ for both) and no difference to the non-ischemic limb.

stimulation on vascular structure had functional implications, we used first-pass magnetic resonance perfusion imaging to assess hind-limb perfusion (Fig. 3*f*). Three weeks after the operations, hind-limb blood flow to the ischemic limb was three-fold higher in the mice treated with nicotine or epibatidine compared with saline-treated mice (Fig. 3*g*). The administration of nicotine or epibatidine to the ischemic hind limb virtually normalized blood flow compared with the contralateral non-ischemic limb. The blood-flow values derived using perfusion imaging were concordant with both the number and size of collateral vessels as well as the capillary density values.

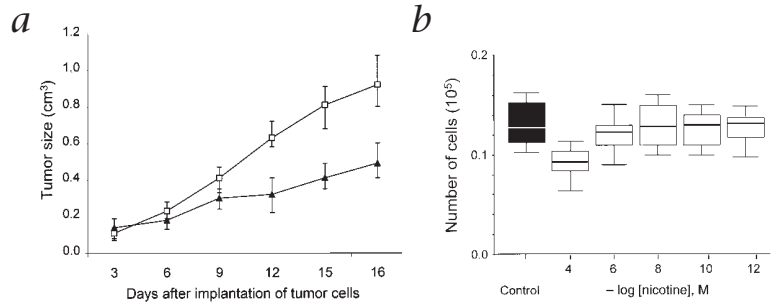

Fig. 4 Nicotine stimulates tumor growth in the Lewis lung cancer model. ***a***, During the first week after subcutaneous injection of Lewis lung cancer cells, tumor growth was similar in the nicotine (□) and the saline group (▲). In the subsequent study period, nicotine-treated mice showed significantly more tumor growth and were killed after 16 days ($P < 0.01$). ***b***, During *in vitro* cancer-cell proliferation studies, nicotine did not change the proliferation rate (*P*-trend = 0.79).

Nicotine stimulates tumor growth

The previous experiments indicated that nicotine was capable of stimulating angiogenesis in different experimental conditions. To determine whether nicotine could enhance tumor angiogenesis, we used the *in vivo* Lewis lung cancer model. Seven days after implantation of the cancer cells and treatment with nicotine or vehicle, all mice had similar tumor size (Fig. 4*a*). At day 16, however, tumor growth in the nicotine group markedly exceeded that in the vehicle-treated group and required killing of the mice. This acceleration of tumor growth in the nicotine group corresponded with increased vascularization of the tumor tissue. At the time of tumor explantation, we observed a significantly higher capillary density in the tumors from mice exposed to nicotine (1.1 (95% CI 0.8–1.7) versus 0.2 (95% CI 0.1–0.4) capillaries/kilopixel ; $P < 0.001$). In another experiment, we

Table 1 Mouse model of atherosclerosis (median (95% CI)).

	Control	Nicotine	Nicotine + rofecoxib	Rofecoxib
	n = 12	*n* = 9	*n* = 11	*n* = 12
Total cholesterol (mg/dl)	1052 (851–1225)	934 (897–1102)	1021 (791–1208)	968 (901–1158)
Bodyweight (g)	26.1 (24.1–28.6)	25.2 (23.8–27.9)	26.7 (22.5–27.5)	27.8 (23.8–29.4)
Serum nicotine (ng/ml)	0	246* (198–289)	223* (174–267)	0
Serum VEGF pg/ml	23.5 (19.1–29.8)	62.9* (53.5–78.2)	35.4 (22.8–44.7)	19.2 (14.1–22.9)
Plaque cell density (cells/mm^2)	812 (617–1212)	941 (652–1231)	912 (704–1118)	837 (598–1021)
SMC content (% of total cells)	22.5 (18.2–25.5)	23.8 (17.1–26.9)	21.2 (20.5.2–23.1)	22.1 (15.9–25.2)
Aortic surface covered with lesions (%)	24.6 (19.2–26.8)	22.8 (18.5–25.5)	24.6 (19.4–27.2)	23.5 (15.9–25.2)

*, $P < 0.01$ versus control

orthotopically implanted Lewis lung cancer cells into the lung parenchyma. Due to the excessive growth of tumors in the nicotine group, mice had to be killed after 12 days (tumor volume of 0.51 cm^3 (95% CI 0.17–0.73) versus 0.22 cm^3 (95% CI 0.18–0.45); $P < 0.001$). Again, tumor vascularization was significantly higher in the nicotine group (1.8 capillaries/kilopixel (95% CI 0.9–2.5) versus 0.5 capillaries/kilopixel (95% CI 0.2–0.9); $P < 0.001$). Moreover, the systemic levels of VEGF were significantly higher in the nicotine group compared with control (54.1 (95% CI 38.3 – 69.8) versus 197.9 (95% CI 170.0 – 225.7) pg/ml; $P < 0.001$).

In subsequent cell-culture experiments, we found that nicotine did not have a direct effect on proliferation of Lewis lung cancer cells (Fig. 4*b*). Moreover, nicotine did not affect the percentage of cells undergoing apoptosis during hypoxia (data not shown).

Nicotine stimulates growth of advanced atherosclerotic lesions

In a mouse model of atherosclerosis (apolipoprotein (ApoE)-deficient mice), growth of advanced plaques depends upon plaque vascularization. The inhibitor of angiogenesis, endostatin, reduced plaque vascularization and thereby lesion growth in the mouse aorta[19]. These findings indicate that neovascularization of atheroma is required for its progression[19,20]. Similarly, Celletti *et al.* have shown that VEGF enhances atherosclerotic plaque progression[21]. Accordingly, we investigated whether nicotine promotes the vascularization and growth of atheroma.

Median plaque area at the age of 20 weeks was 0.19 mm^2 (95% CI 0.15 – 0.24) (Fig. 5*a*). At this time point, we treated mice with nicotine (100 µg/ml in drinking water) or vehicle until the age of 40 weeks. At the end of this treatment period, low-density lipoprotein (LDL) cholesterol and body weight of the ApoE-deficient mice were similar in the four groups (Table 1). Three deaths occurred during this period, all in the nicotine group (at weeks 34, 36 and 39). At week 40, median plaque area in the nicotine group was significantly greater than the control group. The acceleration of lesion growth in nicotine-treated mice was abrogated by concomitant administration of rofecoxib (a COX-2 inhibitor that inhibits angiogenesis[22] but does not have direct effects on atherogenesis[23]).

We also examined plaques isolated from the aortic sinus for the presence of intimal vessels. The acceleration of plaque growth in nicotine-treated mice was associated with an increase in plaque vascularization (Fig. 5*b*–*e*). The percentage of vascularized plaques was significantly higher in nicotine-treated mice (46% nicotine-treated versus 18% control; $P < 0.001$). We observed a similar difference in the vessel density. Adjusting for the differences in plaque area, we found significantly more vascular structures in the nicotine group (3.8 (95% CI 1.71–7.95) vessels/mm^2) compared with the control group (1.4 (95% CI 1.00–3.35); $P = 0.025$). When rofecoxib was co-administered with nicotine, plaque vascularization in the nicotine group was reduced to the level of the control group. We identified vessels in 21% of the sections ($P = 0.72$ versus control) and the vessel density was similar to that of the control group (1.3 (95% CI 1.00–2.54); $P = 0.92$ versus control). Mice treated with rofecoxib alone showed a reduction in plaque vascularity with vascular structures in 5% of the sections ($P = 0.015$ versus control).

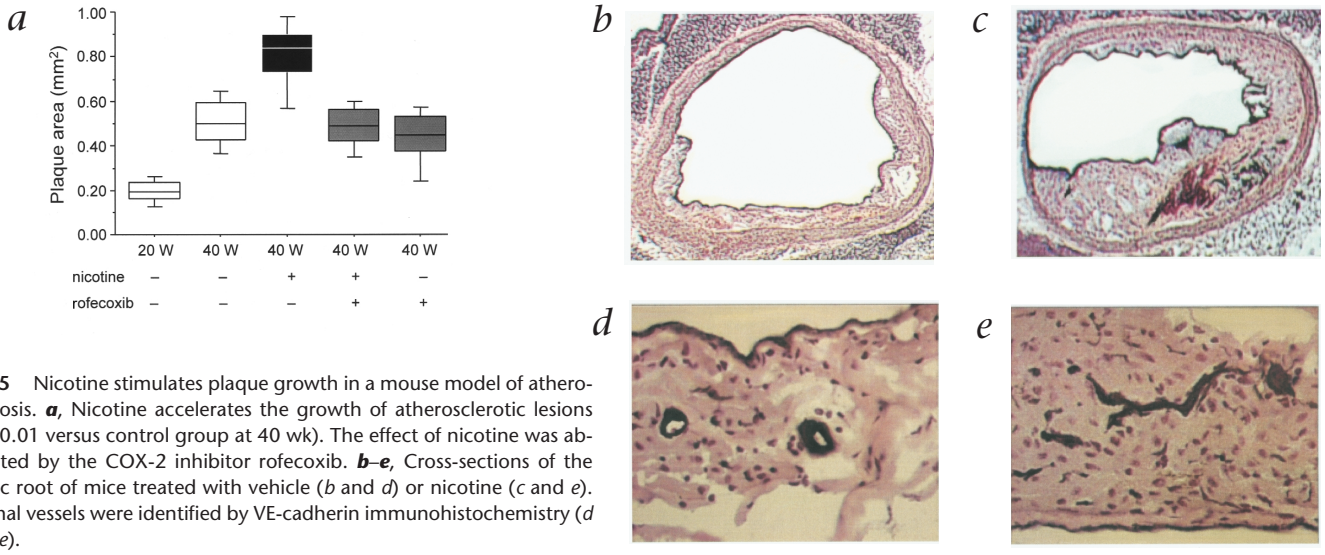

Fig. 5 Nicotine stimulates plaque growth in a mouse model of atherosclerosis. ***a***, Nicotine accelerates the growth of atherosclerotic lesions ($P < 0.01$ versus control group at 40 wk). The effect of nicotine was abrogated by the COX-2 inhibitor rofecoxib. ***b***–***e***, Cross-sections of the aortic root of mice treated with vehicle (*b* and *d*) or nicotine (*c* and *e*). Intimal vessels were identified by VE-cadherin immunohistochemistry (*d* and *e*).

We also investigated the influence of nicotine on SMC migration and proliferation in atherosclerotic lesions (Table 1). The median SMC content of aortic sinus lesions from control mice was 20.4% and was similar for nicotine-treated mice (19.4%; $P = 0.20$). These results are consistent with our hypothesis that nicotine stimulates growth of advanced atherosclerotic lesions by increasing lesion vascularization rather than directly promoting vascular cell growth[24,25].

Discussion

Here we show that nicotine increases endothelial cell number, reduces apoptosis and increases capillary network formation *in vitro*; that nicotine enhances the angiogenic response to inflammation (disc angiogenesis model), ischemia (femoral artery ligation model), atherosclerosis (ApoE-deficient mice) and neoplasia (Lewis lung cancer model); that the effect of nicotine on vascular structure is associated with functional changes in tissue blood flow and tissue growth; and that the proangiogenic effects of nicotine are mediated by non-neuronal nAChR and might involve the elaboration of nitric oxide, prostacyclin and VEGF.

We also show for the first time that nicotine induces angiogenesis. Previous work by Villablanca provided *in vitro* evidence that nicotine stimulates endothelial-cell proliferation[4]. Here we support these findings by showing that endothelial-cell proliferation is involved in the angiogenic response. Additionally, we found that nicotine reduces endothelial-cell apoptosis and causes morphological changes in the endothelial monolayer identical to those induced by VEGF. Specifically, both agents cause endothelial cells to align themselves into whorls on a two-dimensional matrix. Our *in vivo* studies demonstrated that nicotine induced angiogenesis in a number of settings, including inflammation, ischemia, tumor and atherosclerosis. The magnitude of the angiogenic effect of nicotine in these models is comparable with that of angiogenic factors such bFGF. The nicotine-enhanced vascularization was associated with accelerated growth of tumors and atherosclerotic lesions. Nicotine might therefore accelerate tumor growth and atherosclerosis at least in part by increasing angiogenesis.

Antagonists of the nAChR abolished the proangiogenic effect of nicotine. Recent patch-clamp studies demonstrated that human endothelial cells express functional nAChR (ref. 3). Acetylcholine is synthesized and stored in endothelial cells, indicating that acetylcholine might act as an autocrine factor in the vascular system[26,27]. Nicotine might act through this receptor to stimulate endothelial processes involved in angiogenesis. In this respect, we found that the synthesis of nitric oxide was required for the angiogenic effect of nicotine, as the NOS inhibitor L-nitroarginine blocked nicotinic angiogenesis. Recent *in vivo* studies have shown that nicotine might increase production of and response to NO, in bypass grafts[28]. Nicotine increased expression[5] and activity[29] of eNOS *in vitro*. In our studies, the angiogenic effect of nicotine was also blocked by indomethacin or rofecoxib indicating that a product of COX is involved. Nicotine releases prostacyclin from human vascular endothelial cells[10]. Several lines of evidence indicate that prostacyclin is involved in angiogenesis, and might contribute to the angiogenic effects of VEGF (refs. 30,31). We also found that nicotine increased serum levels of VEGF.

There are other potential mechanisms by which nicotine might enhance angiogenesis *in vivo*. Nicotine promotes the release of bFGF and endothelin from endothelial cells[6,8,32,33]. An increased expression of matrix metalloproteinases has been reported to occur in response to nicotine[6]. This might be significant in that dissolution of extracellular matrix is necessary for endothelial-cell migration during angiogenesis[34]. It is also possible that nicotinic stimulation of tissue cells could alter their metabolic needs, and thereby indirectly influence angiogenic response. Alternatively, nicotine is known to influence leukocyte function, and could thereby directly stimulate circulating blood elements (for example, monocytes[35,36]) that have a role in angiogenesis[37]. Moreover, nicotine is known to modulate the phenotype and enhance the proliferation of vascular SMCs (refs. 7,38)—processes that could affect angiogenesis or remodeling of collateral channels.

Because tobacco smoke is a complex mixture of over 4,000 chemical constituents, it is difficult to predict the effect of nicotine delivered via the use of tobacco. Whereas we show that nicotine has a potent angiogenic effect, there are several molecules in cigarette smoke that are toxic to endothelial cells (for example, cadmium and reactive oxygen species)[11]. Indeed, Magers *et al.* recently showed that female hamsters exposed to tobacco smoke for 30 days manifested an impairment of angiogenesis in their reproductive organs[39]. Melkonian *et al.* reported that solutions containing components of tobacco smoke induced an abnormal patterning of blood vessels on chick chorioallantoic membranes (CAM) and altered the composition of the extracellular matrix in the CAM mesoderm[40]. The net effect of cigarette smoke on endothelial function might be quite different from that of nicotine alone.

In our studies, nicotine accelerated the growth of aortic atheroma, and increased plaque vascularization. This was inhibited by rofecoxib, a specific COX-2 inhibitor with anti-angiogenic properties[22,23]. These findings are consistent with the hypothesis that plaque progression requires neovascularization. However, smoking and/or nicotine might also accelerate atherosclerosis in other ways: by altering the shear forces and rheology at the endothelial surface; by upregulating endothelial adhesion molecules; by increasing monocyte adhesion and migration into the subintimal space; by augmenting platelet reactivity and the plasma level of fibrinogen; by inducing vasoconstriction; and by increasing endothelial permeability to lipids and other blood components[11–13].

Our findings might have implications for therapeutic uses of nicotine. Currently, nicotine replacement therapy is used to assist in tobacco cessation. The use of transdermal nicotine did not increase ischemic events[41]. This might be because a greater percentage of these patients cease using tobacco and the course of nicotine replacement therapy was brief. However, more chronic uses of nicotine are being contemplated. Agonists of the nAChR are being studied for their beneficial effects in some neurological disorders including Alzheimer disease and chronic pain. Consideration of chronic nicotine therapy must take into account recent information that certain metabolites of nicotine might be carcinogenic[42], together with the knowledge that oncogenesis and angiogenesis are inextricably linked[43]. Although long-term exposure of healthy rats to nicotine does not alter cardiovascular structure or vascular function [24,25], our findings raise concerns about the effect of chronic nicotine use in humans at risk for pathological angiogenesis. In conclusion, the angiogenic effects of nicotine might have a significant role in pathological angiogenesis associated with tobacco-related disorders.

Methods

Cell proliferation. HUVECs were grown in M199 (10% FBS), HCAEC in EGM-MV (endothelial cells from BioWhittaker, Walkersville, Maryland), and Lewis

lung cancer cells (3LL; ATCC, Manassas, Virginia) in RPMI 1640 (10% FBS). Nicotine-free base (1×10^{-14} to 1×10^{-4} M; Sigma) was added to medium containing 0.1% FBS. Cell proliferation was determined by a tetrazolium salt-based cell counting kit (Wako, Richmond, Virginia). Cell apoptosis was detected by flow cytometry with Alexa Fluor 488 annexin V and propidium iodide fluorescence staining (Molecular Probes, Eugene, Oregon).

Collagen gel assay. Laminin (0.5 mg/ml;), type I collagen (1.5 mg/ml; Becton Dickinson, Franklin Lakes, New Jersey), 10× M199, and water were mixed on ice and the pH was adjusted to 9.5 (ref. 15). Endothelial cells were seeded on the gel (1.2×10^6 cells/well) and 2 h later treated with nicotine (1×10^{-14} to 1×10^{-4} M), VEGF (50 ng/ml), hexamethonium (1×10^{-4} M), nicotine + hexamethonium or vehicle. Tube formation was assessed by light microscopy at 24 and 48 h.

Disc angiogenesis system. Discs of polyvinyl alcohol sponge (Rippey, El Dorado Hills, California) were covered with nitrocellulose filters (Millipore, Burlington, Massachusetts). A pellet, cut from the center of the disc, was loaded with a 20-μl solution of nicotine (1×10^{-4} M), bFGF (10 μg/ml), or saline and coated with ethylene-vinyl acetate co-polymer (Dupont, Wilmington, Delaware). The discs were subcutaneously implanted in the back of C57BL/6J. mice ($n = 5$ per group; Jackson, Bar Harbor, Maine). 2 wk later, we infused space-filling fluorescent microspheres to visualize neovascularization of the disc (0.2 μm, Molecular Probes). Paraffin cross-sections (5 μm) were also stained with toludine blue to quantify the area of fibrovascular growth[16].

Mouse model of hind limb ischemia. In 24-wk-old C57BL6J. mice, the distal external iliac artery and the superficial and deep femoral artery were ligated and excised ($n = 6$ mice per group)[17]. Nicotine was administered for 3 wk either by daily intramuscular injection or orally (100 μg/ml) in 2% saccharin. Serum cotinine levels, an indirect indicator of exposure to nicotine, were 216.5 ng/ml (95% CI 189.8–236.2) (STC Technologies, Bethlehem, Pennsylvania)[14]. Serum VEGF levels were determined with a mouse VEGF ELISA kit (R&D Systems, Minneapolis, Minnesota). Magnetic resonance first-pass perfusion imaging was performed with a multi-section, fast imaging technique using a 1.2-cm diameter surface coil and a Signa 1.5 T scanner (GE medical system, Milwaukee, Wisconsin)[44]. Gadopentetate dimeglumine (0.02 mM/kg; Berlex, Richmond, California) was injected into the left jugular vein. Signal intensity versus time curves were plotted by spatially averaging the signal intensities over the regions of interest[44]. The flow index was defined as the ratio between blood flow in the non-ischemic and the ischemic leg. Capillary density was determined in 10-μm cryostat sections of the adductor and semi-membranous muscles. Capillaries were identified by CD31 immunohistochemistry (PharMingen, San Diego, California) and myocytes were counterstained with eosin (Sigma). Capillary density is expressed as a ratio of capillaries to myocytes. Collateral arteries were defined as vessels with a diameter greater than 0.02 mm and positive for both CD31 (endothelial cells) and α-actin (SMCs; Chemicon, Temecula, California).

Lewis lung cancer model. Lewis lung carcinoma cells (1×10^6 cells/mouse) were subcutaneously injected into each flank or percutaneously introduced into the lung parenchyma of C57BL6J wild-type mice. Mice received nicotine (100 μg/ml) or vehicle in the drinking water. Tumor volume was calculated as the product of length × width × 0.5 cm^3. When mice were bearing tumors greater than 1.0 cm^3, the experiment was stopped and all mice were killed for histological evaluation. Tumor vessels were identified by CD31 immunohistochemistry and tumor cells counterstained with eosin.

Mouse model of atherosclerosis. ApoE-deficient mice, fed a 0.15% cholesterol diet (Harlan Teklad, Madison, Wisconsin), were divided into 4 groups ($n = 12$ each) at the age of 20 wk. Mice were treated for 20 wk with nicotine (100 μg/ml drinking water), nicotine + rofecoxib; rofecoxib only, or placebo. Rofecoxib, an inhibitor of COX-2 and angiogenesis[22], was given in a daily dose of 3.0 mg/kg. We analyzed lesion size and vascularity in the atheroma of the aortic root using acetone-fixed cryostat sections (6 μm)[45]. Intimal vessels and SMCs were identified by VE-cadherin (PharMingen) and α-actin immunohistochemistry, respectively. We verified the specificity of the VE-cadherin antibodies using adjacent, non-vascularized sections that were negative for CD31, vWF and CD146 (ref. 46). In such sections, the endothelium of the conduit vessel stained positive whereas the interior of the atherosclerotic plaque was also negative for VE-cadherin.

Statistical analysis. Continuous variables are expressed as medians with 95% confidence intervals (figures: 75 and 95% confidence intervals). Comparisons between groups were analyzed by *t*-test (two-sided) or ANOVA for experiments with more than two subgroups. *Post hoc* range tests and pair-wise multiple comparisons were performed with the *t* test (two-sided) with Bonferroni adjustment. Comparison of categorical variables was generated by the Pearson χ^2 test. All analyses were performed with SPSS 10.0 (SPSS, Chicago, Illinois). *P* values less than 0.05 were considered statistically significant.

Acknowledgments

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