Journal of Cellular Biochemistry 97:1370–1378 (2006)

Cellular and Molecular Mechanisms of Nicotine's Pro-Angiogenesis Activity and Its Potential Impact on Cancer

Sarah Mousa and Shaker A. Mousa*

Pharmaceutical Research Institute, Albany College of Pharmacy, Albany, New York

Abstract The present study examined the mechanisms of nicotine's effect on angiogenesis and its impact on tumor growth. Nicotine demonstrated significant ($P < 0.01$) stimulation of the release of endothelial cell growth factor, basic fibroblast growth factor (b-FGF) but not vascular endothelial growth factor (VEGF). In a concentration-dependent manner, nicotine induced endothelial cell tube formation. Additionally, in the chick chorioallantoic membrane (CAM) model of angiogenesis, nicotine effectively induced the generation of new blood vessels ($P < 0.01$), an effect that is mediated via b-FGF. The pro-angiogenesis effect of nicotine in the CAM model was maximally blocked by either anti-integrin $\alpha_v\beta_3$ or inhibitor of mitogen activated protein kinase (MAPK, ERK 1/2). In the CAM tumor implant model, nicotine doubled ($P < 0.01$) the growth rate of breast, colon, and lung cancer. These data indicated that the pro-angiogenesis effect is mediated via b-FGF and induced through the nicotinic receptor, $\alpha_v\beta_3$ integrin, and MAPK. J. Cell. Biochem. 97: 1370–1378, 2006. © 2005 Wiley-Liss, Inc.

Key words: angiogenesis; dermal fibroblast; endothelial cell; fibroblast growth factor; nicotine; tumor growth

The epidemic of lung cancer and chronic degenerative diseases associated with tobacco smoking has represented one of the most dramatic catastrophes of modern times [Alberg and Samet, 2003]. Tobacco smoke is known to increase the incidence of cancer, mainly because of tar and other carcinogens in tobacco [Miller et al., 2003]. Tar, a compound in tobacco, is made up of dozens of chemicals, 40 of which are carcinogens [Smith et al., 1997]. Nicotine, another one of the many substances in tobacco, is addictive, which makes smoking so hard to quit [Dani and De Biasi, 2001]. Once used as an insecticide, nicotine is no longer sold as such because of its toxicity [Casanova et al., 2002].

Tobacco smoke is known to promote mutagenesis and carcinogenesis [De Flora et al., 2003]. Earlier studies showed that nicotine increases DNA synthesis and proliferation of vascular endothelial cells in vitro [Villablanca, 1998]. Additionally, nicotine and tobacco-related nitroamines were shown to modulate the growth of human cancer cell lines in vitro [Schuller, 1989]. Hence, this study was designed to examine the effect and mechanism of nicotine on angiogenesis and various angiogenesis-mediated processes.

Angiogenesis, the generation of new blood vessels from pre-existing ones, is required for invasive tumor growth and metastasis; endothelial cells play a key role in this process [Folkman, 1995, 2002; Ibukiyama, 1996]. Avascular tumors are severely restricted in their growth potential because of the lack of blood supply [Folkman, 1995, 2002; Ibukiyama, 1996]; tumors can not grow beyond 1–2 mm without angiogenesis. For tumors to develop in size and metastatic potential, they must make an "angiogenic switch" through perturbation of the local balance of pro-angiogenic and anti-angiogenic factors. Frequently, tumors over-express pro-angiogenic factors, such as basic fibroblast growth factor (b-FGF), allowing them to make this angiogenic switch [Folkman, 1995, 2002; Ibukiyama, 1996].

*Correspondence to: Shaker A. Mousa, PhD, MBA, FACC, FACB, Pharmaceutical Research Institute, Albany College of Pharmacy, 106 New Scotland Avenue, Albany, NY 12208. E-mail: mousas@acp.edu

Received 3 October 2005; Accepted 28 October 2005

DOI 10.1002/jcb.20741

© 2005 Wiley-Liss, Inc.

Endothelial cells are the pivotal cellular components of the angiogenic process and respond to many cytokines through cell surface receptors and intracellular signaling mechanisms [Auerbach et al., 1974]. In culture, endothelial cells are capable of forming tube-like structures with a lumen [Grant et al., 1991]. Therefore, endothelial cells are not only a prerequisite for neovascularization but are also the basal structural requirement [Auerbach et al., 1974; Grant et al., 1991]. Excessive or pathological angiogenesis is implicated in tumor growth, certain ocular diseases, and inflammatory disorders. In contrast, insufficient angiogenesis is implicated in diseases associated with insufficient blood supply to different organs including the skin (wound healing), heart (acute heart attack), brain (stroke), and other organ-related vascular disorders.

The present study was undertaken to determine the effects of nicotine on human endothelial cell tube formation, on angiogenesis, and on angiogenesis-mediated processes, such as the growth rate of various tumors.

MATERIALS AND METHODS

Reagents

Nicotine, mecamylamine, and other chemical-grade reagents were purchased from the Sigma Company (St. Louis, MO). PD 98059 was obtained from Calbiochem (La Jolla, CA). The growth factors, b-FGF and vascular endothelial growth factor (VEGF), were obtained from Invitrogen (Carlsbad, CA). Polyclonal anti-b-FGF and other IgG antibodies such as against $\alpha_v\beta_3$ integrin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

Human umbilical vein endothelial (HUVE) cells or human cancer cell lines were obtained from ATCC (Rockville, MD). HUVE cells were grown to 80%–90% confluence in endothelial growth medium (EGM) containing hEGF 10 ng/ml, hydrocortisone 1 mg/ml, gentamicin 50 mg/ml, amphotercin-B 50 μg/ml, blood–brain extract 0.012 mg/ml, and 2% fetal bovine serum equilibrated with 95% air/5% CO_2 at 37°C. HUVE cells were serially passed in endothelial growth medium in cell culture flasks coated with 0.2% gelatin (Sigma). Confluent cultures of endothelial cells between the third and sixth passages were washed with HBSS and harvested using 0.025% trypsin/0.01% ethylene diamine tetracetate (EDTA) and counted by hemocytometer. Endothelial cells were resuspended in 24-well plates coated with Matrigel matrix or directly on 96-well cell culture plates; these cells were incubated in basal media or growth media.

Endothelial Cell Tube Formation Assay

The effect of nicotine on human endothelial cell tube formation in comparison to b-FGF was examined using HUVE cells. Matrigel growth factor reduced (GFR) obtained from Becton Dickinson (Bedford, MA) was thawed overnight at 4°C. Using cold pipette tips, 250 μl of Matrigel GFR was placed in a cold 24-well plate. Matrigel GFR was allowed to polymerize during incubation at 37°C for 30 min. Endothelial cells were trypsinized, centrifuged, and subsequently washed twice in phosphate buffered saline (PBS). After counting, HUVE cells were plated at 40,000 cells/well in EBM containing b-FGF or nicotine in a 24-well plate. Plates were incubated overnight at 37°C, 5% CO_2 and 95% O_2. Subsequently, the media were removed, and cells were fixed and stained using a modified Hema 3 Stain kit (Fisher; Swedesboro, NJ). Endothelial cell tube formation assay (briefly described above) was carried out according to the detailed method described by Grant et al. [1991].

Microscopic Analysis of Endothelial Tube Formation

Digital images of microtiter well plates were collected using a 3-CCD color video camera system obtained from Sony America (New York, NY) and analyzed with the Image-Pro Plus software obtained from Media Cybernetics (Silver Spring, Maryland). The area and major axis length of stained cells having a tubular morphology on the Matrigel surface was counted from 5 images/well. The effect of nicotine or b-FGF at 0.1, 1.0, and 10 μg on endothelial cell tube formation was determined. The effect of b-FGF polyclonal antibody on nicotine-induced endothelial cell tube formation was also examined.

Endothelial Cell Growth Factor Release

HUVE cells were incubated in 24-well plates with cell-culture medium. The cell medium was sampled at 24 h after incubation with different

concentrations of nicotine. The endothelial cell-released angiogenic factors b-FGF and VEGF in the media were assayed using specific immunoassay ELISA techniques as described by Edgren et al. [1999].

Angiogenesis in the CAM Model

In vivo neovascularization was examined by the method previously described by Auerbach et al. [1974] and Mousa et al. [2005]. The pro-angiogenesis efficacy of nicotine in comparison to the standard pro-angiogenesis growth factors b-FGF or VEGF was determined using the chick chorioallantoic membrane (CAM) model of angiogenesis. Ten-day-old embryos were purchased from Spafas, Inc. (Preston, CT) and incubated at 37°C with 55% humidity. A hypodermic needle was used to puncture a small hole in the area of the shell concealing the air sac. A second hole was punctured in the shell on the broadside of the egg directly over an avascular portion of the embryonic membrane. A false air sac was created beneath the second hole by the application of negative pressure to the first hole, which caused the membrane to separate from the shell. A window, approximately 1.0 cm^2, was cut in the shell over the dropped CAM with the use of a small crafts grinding wheel, which allowed direct access to the underlying CAM. Figure 1 is a diagrammatic sketch showing the steps involved in the CAM model. Filter disks were soaked in 3 mg/ml cortisone acetate and subsequently air dried under sterile conditions. The pro-angiogenic growth factor b-FGF was used as the stimulant of angiogenesis on the CAMs of 10-day-old chick embryos. This experiment involved a control (PBS), a positive control (b-FGF), and treatment groups with b-FGF or nicotine at different doses. Additionally, the effect of b-FGF polyclonal antibody, nicotinic receptor antagonist, $\alpha_v\beta_3$ integrin antagonists, or MAP kinase (ERK 1/2) inhibitor on nicotine-induced angiogenesis was examined in the CAM model.

Sterile filter disks adsorbed with b-FGF, nicotine, or nicotine plus b-FGF antibody dissolved in PBS were placed on growing CAMs. CAMs were harvested on the fourth day of stimulation (Fig. 1). CAM tissue directly beneath the filter disk was removed from embryos, and tissues were washed three times with PBS. Blood vessel branch points in the 5-mm filter disk area were counted at 30× magnifications as a quantitative indicator of vascular sprouting in response to growth factors. The number of vessel branch points contained in a circular region equal to the area of the filter disk was counted for each section. Because angiogenesis is characterized by the sprouting of new vessels in response to growth factors, counting blood vessel branch points is a useful quantitative means to determine a compound's angiogenesis efficacy.

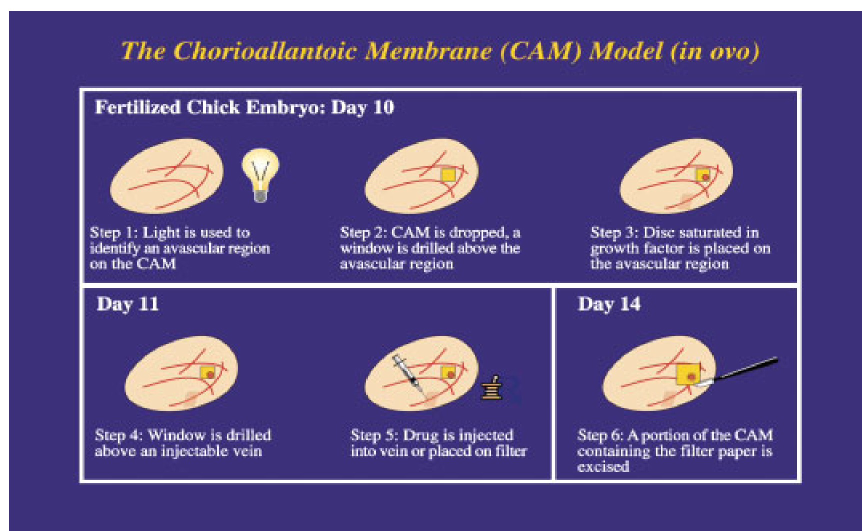

Fig. 1. Protocol for the CAM angiogenesis model. Illustration of the steps (1–6) involved in carrying out experiments with pro-angiogenesis or anti-angiogenesis agents. The protocol is carried out with fertilized chick embryos at day 10 until day 14. The number of CAMs per group in each experiment ranges from 8–10, and each experiment is repeated three times. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Tumor Growth in the CAM Tumor Implant Model

The effect of nicotine on different types of tumor growth (weight) was determined after 1 week of 1×10^6 breast, colon, and lung cancer cells were implanted in the CAM tumor model, as described [Mousa et al., 2005]. Tumor cells (breast, colon, or lung) at 10^6 cells/CAM were placed on the surface of each CAM (7-day-old embryo), with the membrane dropped, as described in the in vivo angiogenesis model, for 1 week. The effect of nicotine at 1.0 μg on the growth of those tumor types was determined. Tumors were then removed from the egg, and weights were determined for each tumor. Data are presented as mean tumor weight per treatment group ± SD, n = 8 per group.

Statistical Analysis

Statistical significance for data presented in these experiments was analyzed by the Student's *t*-test. Data were compared using Student's *t*-test for paired comparison between control and treated groups. $P < 0.01$ or less reflects statistically significant differences within a confidence limit of >99%.

RESULTS

Effect of Nicotine on Human Endothelial Cell Tube Formation

Nicotine increased ($P < 0.01$) human endothelial cell tube formation in a concentration-dependent manner (Fig. 2). Nicotine demonstrated endothelial cell tube formation effect comparable to that observed with b-FGF (Fig. 2). The effect of nicotine on endothelial cell tube formation was totally blocked by b-FGF polyclonal antibody (Fig. 3).

Nicotine and Endothelial Cell Growth Factor Release

The effect of nicotine on the release of endothelial cell growth factors including b-FGF and VEGF was examined. Data demonstrated differential stimulation of endothelial b-FGF release but not of VEGF (Fig. 4).

Efficacy and Mechanisms of Nicotine on Angiogenesis in the CAM Model

The protocol used is as shown in Figure 1. Nicotine demonstrated comparable stimulation of angiogenesis to b-FGF or VEGF in the CAM model (Fig. 5a). Nicotine increased significantly ($P < 0.01$) new blood vessel formation from existing ones in a dose-dependent manner (Fig. 5b).

The pro-angiogenesis effect of nicotine was totally blocked by b-FGF polyclonal antibody (Fig. 6a). These data suggest the mediation of the pro-angiogenesis effect of nicotine via b-FGF. Additionally, because b-FGF is known to induce angiogenesis via the cell adhesion integrin receptor ($\alpha_v\beta_3$) and MAP kinase (ERK 1/2), the effect of specific inhibitors of $\alpha_v\beta_3$ integrin (monoclonal antibody, LM609) and the MAP kinase inhibitor (PD 98059) was determined. Potent inhibition of nicotine-induced angiogenesis in the CAM model was demonstrated after nicotinic receptor blockade with

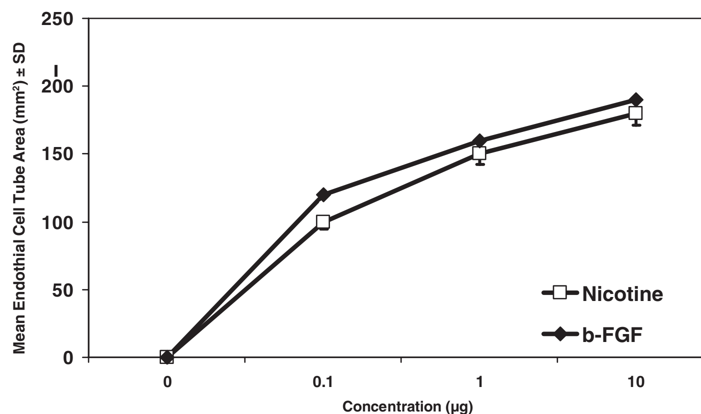

Fig. 2. Comparative effect of nicotine and b-FGF on endothelial cell tube formation. Data plotted is the mean of n = 8, ± SD. A dose-dependent effect of nicotine or b-FGF on endothelial cell tube area was demonstrated, with a maximal effect at 1–10 μg.

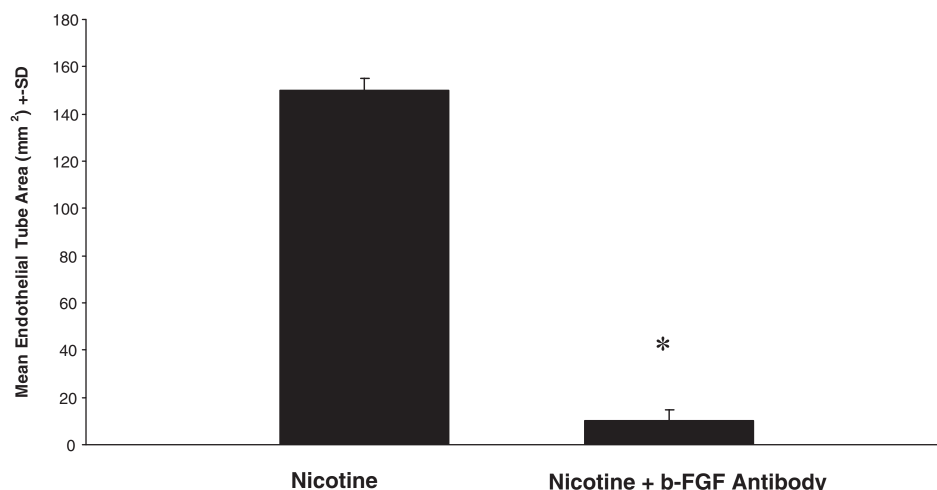

Fig. 3. Effect of b-FGF antibody on nicotine-induced EC tube formation. Data plotted is the mean of n = 8, ± SD. The stimulatory effect of nicotine on human endothelial cell tube formation is significantly blocked by b-FGF antibody. *$P < 0.001$.

mecamylamine or $\alpha_v\beta_3$ integrin blockade or the inhibition of MAP kinase (Fig. 6b,c).

Nicotine and Tumor Growth in the CAM Model

Nicotine at 1.0 μg demonstrated significant ($P < 0.01$) increase in breast, colon, and lung cancer growth rate by at least twofold after 1 week of implant in the CAM model (Fig. 7). This demonstrated the risk to nicotine exposure in cancer patients or those at risk.

DISCUSSION

Nicotine demonstrated potent stimulation of endothelial cell tube formation, as compared with the angiogenic growth factor b-FGF or VEGF. Nicotine was also shown to induce b-FGF but not VEGF release from human endothelial cells. Nicotine effectively induced the generation of new blood vessels from existing ones in the CAM model, and it increased tumor growth in the CAM tumor implant model regardless of the tumor type. The endothelial and angiogenesis stimulating effects of nicotine were shown to be mediated via b-FGF, as evident from the total blockade of nicotine's effects by b-FGF antibody. These data are supported by our initial finding of the differential effect of nicotine in enhancing endothelial b-FGF but not VEGF release, as shown in Figure 4.

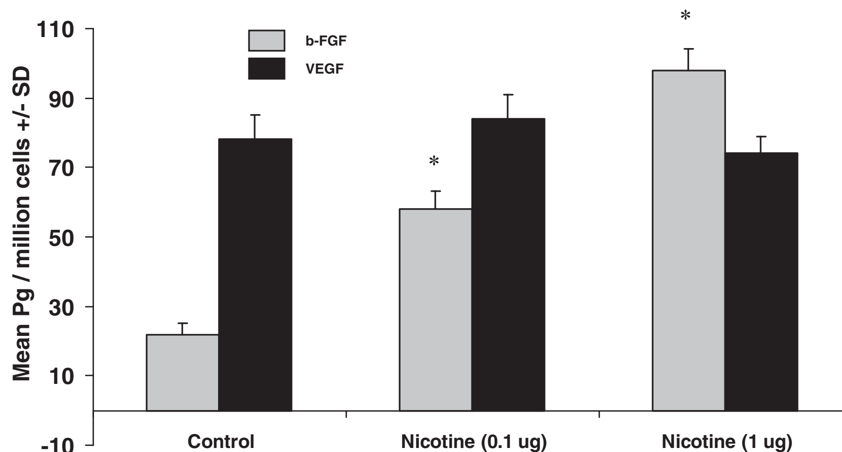

Fig. 4. Effect of nicotine on the endothelial release of basic fibroblast growth factor (b-FGF) and vascular endothelial growth factor (VEGF). *$P < 0.01$ as compared to control (Student's *t*-test). Nicotine at 0.1–1 μg significantly increased the release of endothelial b-FGF but not VEGF after 24 h of incubation.

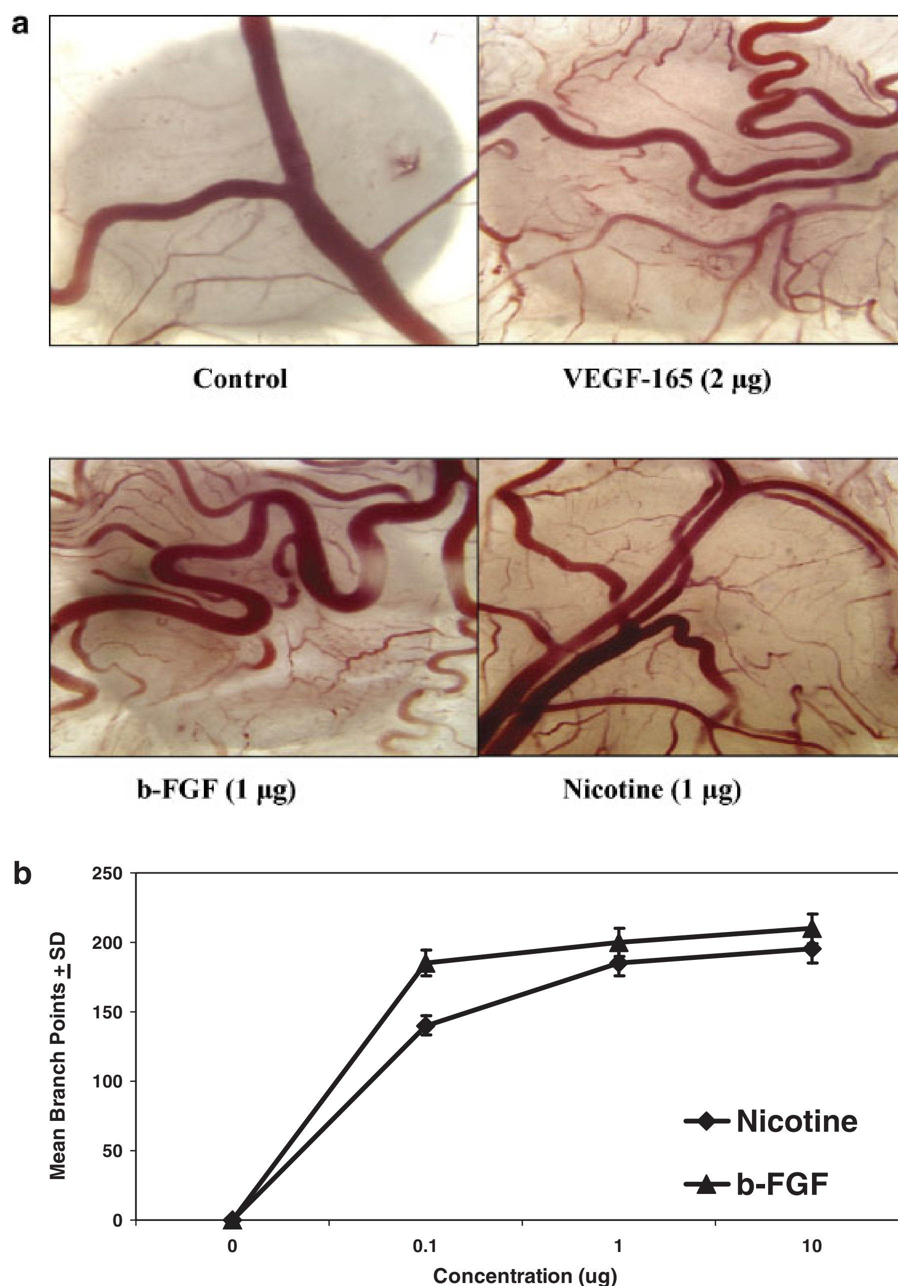

Fig. 5. **a**: Comparative pro-angiogenesis efficacy between nicotine and b-FGF or VEGF. In the CAM model, nicotine demonstrated potent pro-angiogenesis efficacy in promoting new blood vessel branch points, which is comparable to that shown with b-FGF or VEGF. **b**: Comparative effect of nicotine versus b-FGF on angiogenesis in the CAM model. Data plotted is the mean ± SD, n = 8. In the CAM model, either nicotine or b-FGF resulted in a comparable stimulation of angiogenesis, with a maximal effect at 0.1–1 μg. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 6. **a**: Effect of b-FGF antibody on the pro-angiogenesis effect of nicotine in the CAM model. Data plotted is the mean of n = 8, ± SD. In the CAM model, the pro-angiogenesis effect of nicotine is significantly blocked by anti-b-FGF antibody. $*P < 0.01$. **b**: Representative illustration of the inhibitory effect of the nicotinic receptor antagonist, mecamylamine, the $\alpha_v\beta_3$ antagonist, LM609 or the MAP kinase (ERK 1/2) inhibitor, PD 98059, in the CAM model. The pro-angiogenesis effect of nicotine is maximally blocked by either the nicotinic receptor antagonist, $\alpha_v\beta_3$ antagonist, or ERK 1/2 inhibitor. **c**: Blockade of nicotine-induced angiogenesis by nicotinic receptor antagonist, $\alpha_v\beta_3$ antagonists, or MAP kinase inhibitor in the CAM model. Dose of nicotine, 1 μg. $*P < 0.001$, n = 8. Maximal blockade (80%–100%) of the pro-angiogenesis effect of nicotine was demonstrated as shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

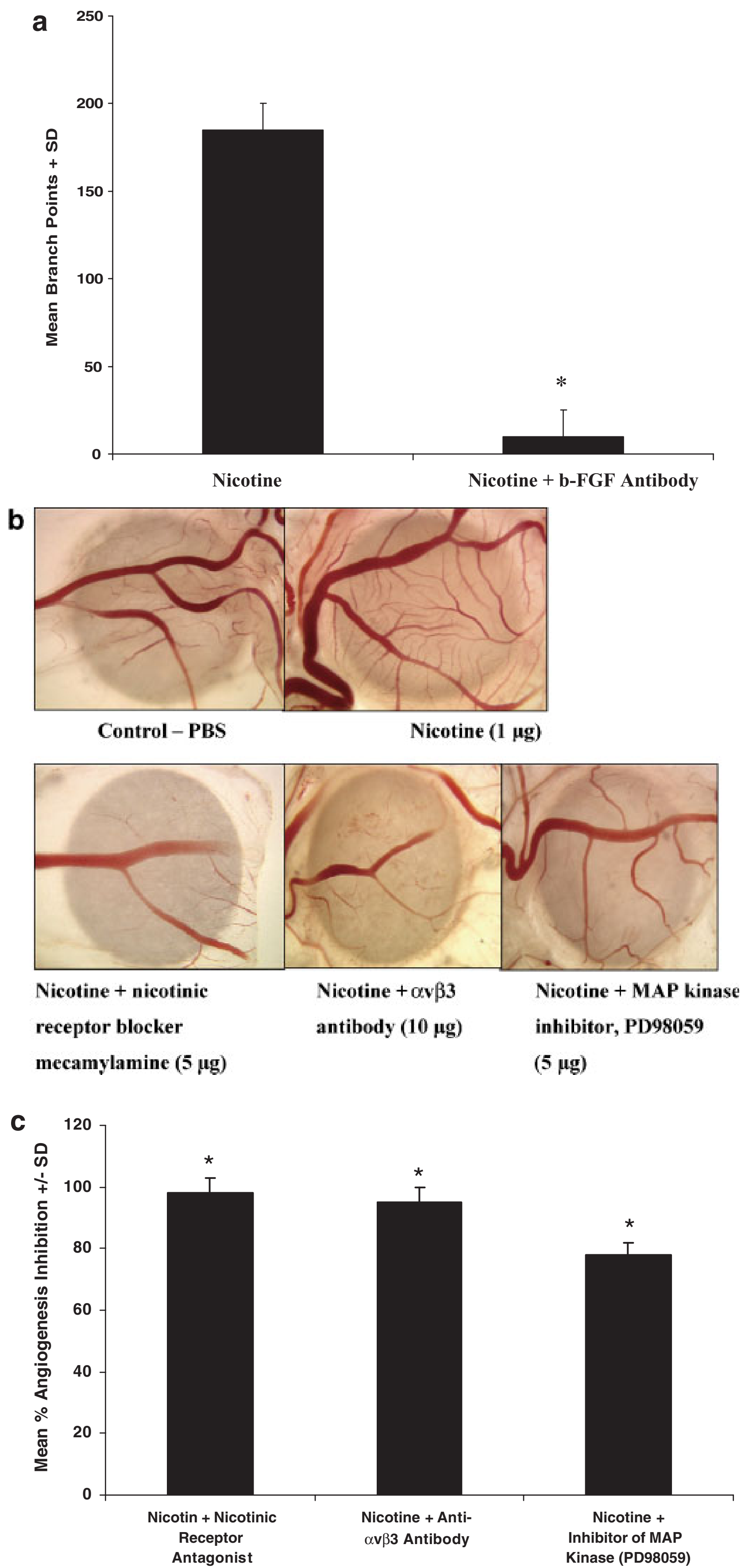

Fig. 6.

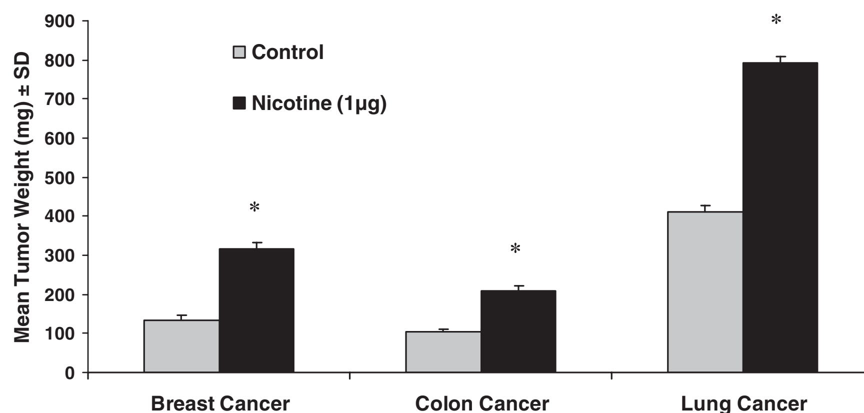

Fig. 7. Effect of nicotine on tumor growth in the CAM model. Data plotted is the mean of n = 8, ± SD. Nicotine significantly enhanced the growth of different tumor types after single exposure of 1 μg/week in the CAM model. *$P < 0.01$.

Endothelial cells express non-neuronal nicotinic receptor [Macklin et al., 1998] as well as the integrin $\alpha_v\beta_3$ receptors [Ruegg et al., 2002]. It is also known that pro-angiogenesis effect of b-FGF but not VEGF is mediated via the integrin $\alpha_v\beta_3$ receptors on endothelial cells [Friedlander et al., 1995], and MAP kinase (ERK 1/2) is involved in the signaling mechanism of b-FGF–$\alpha_v\beta_3$-mediated angiogenesis [Friedlander et al., 1995; Ruegg and Mariotti, 2003]. In that regard, our data clearly showed that the pro-angiogenesis effect of nicotine is mediated via the non-neuronal nicotinic receptor, the $\alpha_v\beta_3$ integrin receptors on cell surface of endothelial cells and is mediated through the intracellular signaling mechanism involving MAP kinase (ERK 1/2).

In agreement with our results, Heeschen et al. [2002] have recently reported that nicotine has angiogenic effects, which appear to be mediated through non-neuronal nicotinic acetylcholine receptors (nAChR). In an in vitro angiogenesis model, increasing concentrations of the non-selective nAChR antagonist mecamylamine completely and reversibly inhibited endothelial network formation. Results from that study suggest that nAChRs may play an important role in physiological and pathological angiogenesis.

CONCLUSION

These data indicated that nicotine has endothelial cell stimulating effects that result in enhanced angiogenesis similar to the effects of standard growth factor such as b-FGF. Nicotine might have a potential adverse effect on cancer patients because of its pro-angiogenesis effects that might promote tumor growth.

In future studies, we would like to examine the effect of nicotine, nicotine derivatives [Mousa and Mousa, 2005] and, in particular, their polymeric forms on wound healing in genetically diabetic animals. Additionally, we would like to further define the molecular effects of nicotine and its polymeric forms on angiogenesis-related genes by cDNA microarray and RT-PCR.

REFERENCES

Alberg AJ, Samet JM. 2003. Epidemiology of lung cancer. Chest 123(1 suppl):21S–49S.

Auerbach R, Kubai L, Knighton D, Folkman J. 1974. A simple procedure for the long-term cultivation of chicken embryos. Dev Biol 41:391–394.

Casanova H, Ortiz C, Pelaez C, Vallejo A, Moreno ME, Acevedo M. 2002. Insecticide formulations based on nicotine oleate stabilized by sodium caseinate. J Agric Food Chem 50(22):6389–6394.

Dani JA, De Biasi M. 2001. Cellular mechanisms of nicotine addiction. Pharmacol Biochem Behav 70(4):439–446.

De Flora S, D'Agostini F, Balansky R, Camoirano A, Bennicelli C, Bagnasco M, Cartiglias C, Tampa E, Longobardi MG, Lubet RA, Izzotti A. 2003. Modulation of cigarette smoke-related end-points in mutagenesis and carcinogenesis. Mutation Res 523–524:237–252.

Edgren M, Lennernas B, Larsson A, Nilsson S. 1999. Serum concentrations of VEGF and b-FGF in renal cell, prostate and urinary bladder carcinomas. Anticancer Res 19(1B):869–873.

Folkman J. 1995. Angiogenesis in cancer, vascular, rheumatoid, and other diseases. Nat Med 1:27–31.

Folkman J. 2002. Role of angiogenesis in tumor growth and metastasis. Semin Oncol 29(6 suppl 16):15–18.

Friedlander M, Brooks PC, Shaffer RW, Kincaid CM, Varner JA, Cheresh DA. 1995. Definition of two angiogenic pathways by distinct alpha v integrins. Science 270(5241):1500–1502.

Grant DS, Lelkes PI, Fukuda K, Kleinman HK. 1991. Intracellular mechanisms involved in basement mem-

brane induced blood vessel differentiation in vitro. In Vitro Cell Dev Biol 27A:327–336.

Heeschen C, Weis M, Aicher A, Dimmeler S, Cooke JP. 2002. A novel angiogenic pathway mediated by non-neuronal nicotinic acetylcholine receptors. J Clin Invest 110(4):527–536.

Ibukiyama C. 1996. Angiogenesis: Angiogenic therapy using fibroblast growth factors and vascular endothelial growth factors for ischemic vascular lesions. Jpn Heart J 37(3):285–300.

Macklin KD, Maus AD, Pereira EF, Albuquerque EX, Conti-Fine BM. 1998. Human vascular endothelial cells express functional nicotinic acetylcholine receptors. J Pharmacol Exp Ther 287(1):435–439.

Miller DP, De Vivo I, Neuberg D, Wain JC, Lynch TJ, Su L, Christiani DC. 2003. Association between self-reported environmental tobacco smoke exposure and lung cancer: Modification by GSTP1 polymorphism. Int J Cancer 104(6):758–763.

Mousa SS, Mousa SA. 2005. Method for treating occlusive vascular diseases and wound healing. United States Patent Application 2005/0069518; March 31, 2005.

Mousa SS, Mousa S, Mousa SA. 2005. Effect of resveratrol on angiogenesis and platelet/fibrin-accelerated tumor growth in the chick chorioallantoic membrane model. Nutr Cancer 52(1):59–65.

Ruegg C, Dormond O, Foletti A. 2002. Suppression of tumor angiogenesis through the inhibition of integrin function and signaling in endothelial cells: Which side to target? Endothelium 9(3):151–160.

Ruegg C, Mariotti A. 2003. Vascular integrins: Pleiotropic adhesion and signaling molecules in vascular homeostasis and angiogenesis. Cell Mol Life Sci 60(6):1135–1157.

Schuller HM. 1989. Cell type specific, receptor-mediated modulation of growth kinetics in human lung cancer cell lines by nicotine and tobacco-related nitrosamines. Biochem Pharmacol 38(20):3439–3442.

Smith CJ, Livingston SD, Doolittle DJ. 1997. An international literature survey of “IARC Group I carcinogens” reported in mainstream cigarette smoke. Food Chem Toxicol 35(10–11):1107–1130.

Villablanca AC. 1998. Nicotine stimulates DNA synthesis and proliferation in vascular endothelial cells in vitro. J Appl Physiol 84(6):2089–2098.