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# Nicotine Enhances Neovascularization and Promotes Tumor Growth

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**Solid tumors require vascularization for their growth. Bone marrow-derived endothelial progenitor cells participate in tumor angiogenesis. Here, we show that nicotine markedly accelerated growth of colon cancer cells inoculated subcutaneously in mice but had no effect on proliferation of carcinoma cells *in vitro*. We found that the tumor growth was associated with increased vascularization of the tumor and that bone marrow-derived cells contributed to the formation of the new blood vessels. Our findings show that nicotine promotes tumor growth, at least in part, by stimulating tumor-associated neovascularization.**

## Introduction

Cigarette smoking is the most important risk factor for cardiovascular disease and cancer, both of which are major causes of death in Western countries (Bartecchi *et al.*, 1994). In particular, 85% of lung cancers are attributable to smoking. Smoking also increases the risk of cancers of other organs, including mouth, pharynx, larynx, esophagus, stomach, pancreas, uterine cervix, kidney, ureter and bladder. However, the mechanism by which smoking increases cancer morbidity remains unknown.

Solid tumors require neovascularization for their growth (Folkman; 1990; 1995; Heeschen *et al.*, 2001; Lyden *et al.*, 2001; O'Reilly *et al.*, 1994). Here we show that nicotine, a major component of cigarette smoke, dramatically accelerates the growth of colon cancer cells inoculated into the subcutaneous space of mice. Our findings indicate that nicotine promotes tumor growth, at least in part, by enhancing tumor neovascularization.

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## Materials and Methods

**Study protocol** All protocols involving experimental animals were in accordance with the institutional guidelines for animal care of the University of Tokyo. Male eight-week-old C57BL/6 mice were purchased from SLC Japan (Japan). Either vehicle (0.5% carboxymethylcellulose) or nicotine (20 mg/kg/day) was administered by gavage every day starting five days before tumor inoculation. The mice were anesthetized by intraperitoneal injection of 50 mg/kg of pentobarbital. We suspended $6 \times 10^7$ murine syngeneic colon cancer cells (CMT93, American Type Culture Collection,USA) in 0.1 ml of ECM gel (Sigma, USA) and injected them subcutaneously into the left flank fold of the mice, and measured tumor size with calipers every day. Tumor volume was calculated as width$^2$ × length × 0.52. The mice were killed at 11 days after implantation. The tumors were excised, fixed in methanol and embedded in paraffin. ROSA26 mice that express lacZ ubiquitously were originally purchased from Jackson Laboratory (Bar Harbor, ME). Bone marrow transplantation was performed as described (Sata *et al.*, 2002).

**Measurement of capillary density** Sections (5 μm) were deparaffinized and incubated with a rat monoclonal antibody against murine CD31 (clone MEC13.1, BD PharMingen, USA) (Sata *et al.*, 2001). Antibody distribution was visualized using the avidin-biotin-complex technique and Vector Red Chromogenic substrate (Vector Laboratories, Burlingame, CA), followed by counterstaining with hematoxylin. Capillaries were identified by positive staining for CD31 and morphology. Ten different fields from each tissue preparation were randomly selected, and capillaries counted. Capillary density is expressed as number of capillaries per square millimeter.

**Detection of bone marrow-derived endothelial cells** Tumors were embedded in OCT compound and snap frozen in liquid nitrogen. Frozen sections (5 μm) were fixed in formalin and stained with anti-lacZ rabbit polyclonal antibody (ICN, Aurora, OH) or anti-CD31 rat monoclonal antibody, followed by incubation with FITC-conjugated anti-rabbit Ig and rhodamine-conjugated anti-rat Ig secondary antibodies. Nuclei were counterstained with Hoechst 33258. The sections were observed with a confocal microscope (FLUOVIEW FV300, Olympus, Japan).

***In vitro* cell proliferation assay** CMT93 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. $7 \times 10^4$ cells were cultured per well of a 24-well culture plate in the presence or absence of nicotine for 24 h. and cell numbers counted after trypsinization.

***In vitro* VEGF production** CMT93 cells were cultured in 24-well culture plates in the presence or absence of nicotine for 24 h. VEGF concentration in the supernatant was measured using an anti-mouse VEGF ELISA kit (Quantikine M Colorimetric Sandwich ELISA kit, R&D systems, Minneapolis, MN).

**Statistical analysis** All results are expressed as means ± S.E.M. Means were statistically compared by ANOVA followed by Student's *t*-test. A value of $p < 0.05$ was considered to be significant.

## Results

**Promotion of tumor growth by nicotine** To determine the effect of nicotine on tumor growth, we used a tumor implantation model; $6 \times 10^7$ murine syngeneic colon cancer cells (CMT93) were injected subcutaneously into the left flank fold of C57BL/6 mice, and the mice were treated with either nicotine (20 mg/kg/day, n = 6) or vehicle (n = 6). Tumor growth in the nicotine-treated group markedly exceeded that in the vehicle group ($p < 0.01$) (Fig. 1).

**Effect of nicotine on carcinoma cell proliferation *in vitro*** Next, we investigated whether nicotine directly stimulates proliferation of carcinoma cells. $7 \times 10^4$ CMT93 cells were cultured in the presence or absence of nicotine ($10^{-12}$ to $10^{-4}$ M) for 24 h (n = 4 for each group). We found no significant difference in cell number between the groups (Fig. 2). On the other hand, nicotine ($10^{-4}$ to $10^{-10}$ M) stimulated VEGF production by the CMT93 cells (data not shown) indicating that nicotine might promote tumor growth by enhancing vascularization.

**Effects of nicotine on tumor vascularization** To study the effect of nicotine on cancer-associated angiogenesis, we harvested tumors 11 days after implantation. Anti-

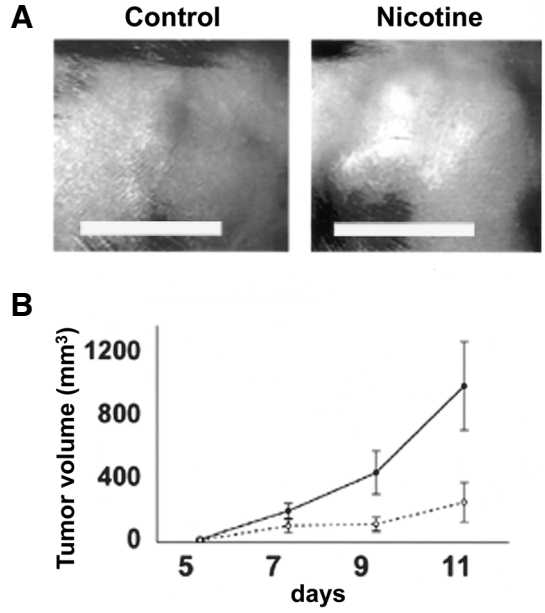


**Fig. 1.** Effect of nicotine on tumor growth *in vivo*. $6 \times 10^7$ murine syngeneic colon cancer cells (CMT93) were implanted subcutaneously into the left flank fold of C57BL/6 mice, and the mice received 20 mg/kg/day of nicotine or vehicle starting 5 days before implantation. **A.** Tumor appearance in the mice treated with nicotine or vehicle. Scale bar, 1 cm. **B.** Nicotine (closed circle, n = 6) or vehicle (open circle, n = 6) was administered to the mice. Tumor size was measured with calipers every day. Tumor volume was calculated as width$^2$ × length × 0.52.

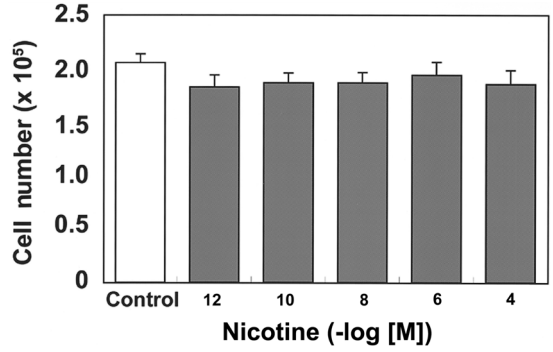


**Fig. 2.** Effect of nicotine on carcinoma cell proliferation *in vitro*. $7 \times 10^4$ CMT93 cells were cultured in the presence or absence of nicotine at the indicated concentrations. Numbers of cells were counted after 24 h (n = 4 for each group).

CD31 immunostaining revealed that many new vessels had formed (Fig. 3A); capillary density in the nicotine-treated mice was significantly higher than in the vehicle group (496 ± 29 versus 269 ± 25 capillaries/mm$^2$, $p < 0.01$) (n = 4 for each group) (Fig. 3B).

To study the potential contribution of bone marrow cells to the capillary formation we inoculated carcinoma cells into wild-type mice whose bone marrow had been replaced with that of ROSA26 mice, and the mice were

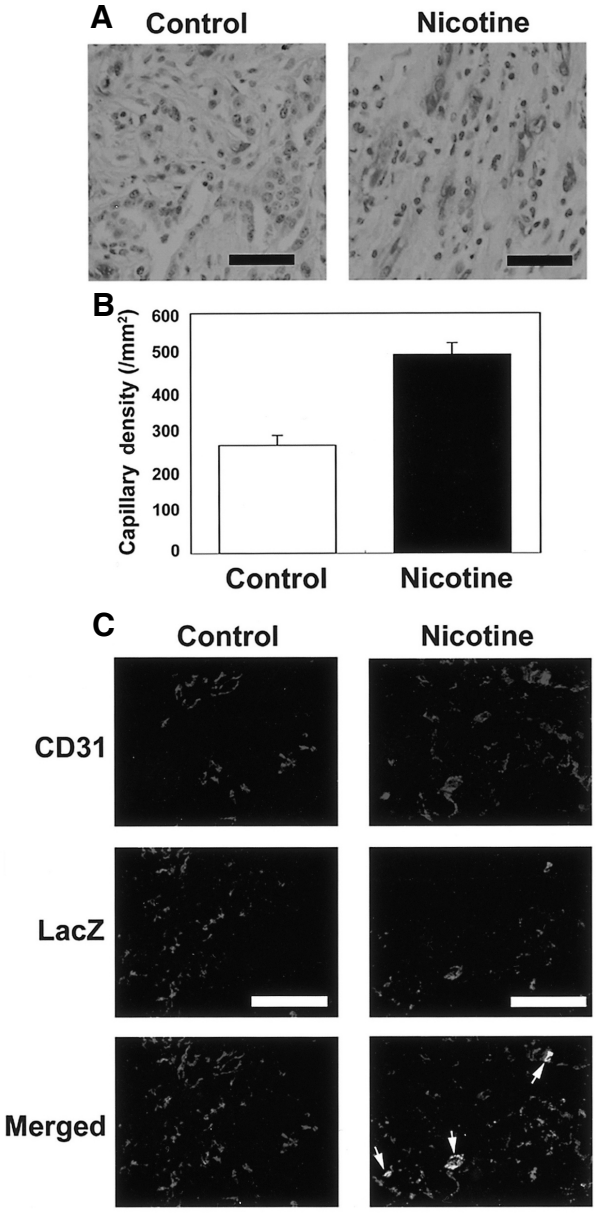


**Fig. 3.** Effect of nicotine on neovascularization in tumors. **A** and **B.** Tumors were harvested 11 days after implantation. Cross sections were stained for CD31 (A). Scale bar, 50 μm. Capillary density was measured (n = 4 for each group) (B). **C.** $1 \times 10^7$ CMT93 cells were implanted into wild-type mice whose bone marrow had been reconstituted with that of ROSA26 mice. The mice received vehicle or nicotine. At day 7, tumors were collected for immunofluorescence double staining to detect CD31 (red) and lacZ (green). Arrows indicate lacZ-positive endothelial cells. Bar, 50 μm.

treated with vehicle or nicotine for 7 d. We found CD31-positive endothelial cells that expressed lacZ, particularly in the tumors of the mice treated with nicotine (Fig. 3C). These results suggest that bone marrow derived cells can contribute to tumor neovascularization.

## Discussion

The nicotine-derived nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1 butanone, is one of the best known carcinogens among the many components of cigarette smoke (Hecht, 1999). On the other hand, it is generally believed that nicotine itself does not cause neoplastic transformation, and we also found that nicotine had no effect on the proliferation of cancer cells *in vitro*. However, it greatly increased the growth of inoculated tumors, and enhanced new blood vessel formation.

The production of new blood vessels involves two processes, vasculogenesis and angiogenesis (Carmeliet, 2000). Vasculogenesis is the *in situ* differentiation of mesodermal precursors to angioblasts that differentiate into endothelial cells to form the primitive capillary network. Angiogenesis is the sprouting of new capillaries from pre-existing blood vessels. Until recently, vasculogenesis was considered to be limited to early embryogenesis and postnatal neovascularization was thought to result from angiogenesis (Folkman and Shing, 1992). However, recent studies have identified circulating endothelial progenitor cells (Asahara *et al.*, 1997; 1999a; 1999b; Peichev *et al.*, 2000; Takahashi *et al.*, 1999) that play a critical role in physiological and pathological neovascularization (Asahara *et al.*, 1999a; Lyden *et al.*, 1999; 2001). In this study, we found that bone marrow-derived cells participated in new vessel development in tumors.

Nicotine acts via the nicotinic acetylcholine receptor (nAChR) that mediates fast synaptic transmission (Pontieri *et al.*, 1996). Endothelial cells also express nAChR and nicotine stimulates their proliferation *in vitro* (Heeschen *et al.*, 2001; Villablanca, 1998). Nicotine also stimulates vascular endothelial cell growth factor (VEGF) expression in endothelial cells (Conklin *et al.*, 2002), and we also found that nicotine stimulated VEGF production in tumor cells and that it enhanced tumor-associated angiogenesis. Taken together, these findings suggest that nicotine up-regulates VEGF expression by cancer cells and augments tumor-associated angiogenesis and vasculogenesis, leading to enhanced tumor growth.

In summary, we found that nicotine promotes tumor growth, at least in part, by stimulating neovascularization. Our findings provide additional insight into the mechanism by which smoking enhances the development of cancers.

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