

Nicotine Induces Cyclooxygenase-2 and Vascular Endothelial Growth Factor Receptor-2 in Association with Tumor-Associated Invasion and Angiogenesis in Gastric Cancer

Vivian Y. Shin,¹ William K.K. Wu,¹ Kent-Man Chu,² Helen P.S. Wong,¹ Emily K.Y. Lam,¹ Emily K.K. Tai,¹ Marcel W.L. Koo,¹ and Chi-Hin Cho^{1,3}

Departments of ¹Pharmacology and ²Surgery and ³Research Centre of Infection and Immunology, Faculty of Medicine, The University of Hong Kong, Hong Kong, Hong Kong

Abstract

Blockade of angiogenesis is a promising strategy to suppress tumor growth, invasion, and metastasis. Vascular endothelial growth factor (VEGF), which binds to tyrosine kinase receptors [VEGF receptors (VEGFR) 1 and 2], is the mediator of angiogenesis and mitogen for endothelial cells. Cyclooxygenase-2 (COX-2) plays an important role in the promoting action of nicotine on gastric cancer growth. However, the action of nicotine and the relationship between COX-2 and VEGF/VEGFR system in tumorigenesis remain undefined. In this study, the effects of nicotine in tumor angiogenesis, invasiveness, and metastasis were studied with sponge implantation and Matrigel membrane models. Nicotine (200 µg/mL) stimulated gastric cancer cell proliferation, which was blocked by SC-236 (a highly selective COX-2 inhibitor) and CBO-P11 (a VEGFR inhibitor). This was associated with decreased VEGF levels as well as VEGFR-2 but not VEGFR-1 expression. Topical injection of nicotine enhanced tumor-associated vascularization, with a concomitant increase in VEGF levels in sponge implants. Again, application of SC-236 (2 mg/kg) and CBO-P11 (0.4 mg/kg) partially attenuated vascularization by ~30%. Furthermore, nicotine enhanced tumor cell invasion through the Matrigel membrane by 4-fold and promoted migration of human umbilical vein endothelial cells in a cocultured system with gastric cancer cells. The activity of matrix metalloproteinases 2 and 9 and protein expressions of plasminogen activators (urokinase-type plasminogen activator and its receptor), which are the indicators of

invasion and migration processes, were increased by nicotine but blocked by COX-2 and VEGFR inhibitors. Taken together, our results reveal that the promoting action of nicotine on angiogenesis, tumor invasion, and metastasis is COX-2/VEGF/VEGFR dependent. (Mol Cancer Res 2005;3(11):607–15)

Introduction

Cigarette smoke has been well documented to be closely related to the development of gastric cancer (1, 2). However, the exact mechanism for the carcinogenic action of cigarette smoke is still largely unknown. Nicotine is recognized to be the major psychoactive compound in cigarette smoke and has been suggested to play a role in carcinogenesis (3). Tumor cell proliferation, angiogenesis, and local and distant cell migration are hallmarks of solid tumors. Our recent studies show that nicotine induces proliferation of human adenocarcinoma cells via a cyclooxygenase (COX)/lipoxygenase-dependent pathway to metabolize fatty acids into eicosanoids (4, 5). The effects of COX-2 inhibitors on treatment and chemoprevention of gastric cancer have been widely reported (6, 7). However, the involvement of COX-2 in tumor invasion and metastasis receives little attention.

Angiogenesis is the growth of new capillaries occurring in many physiologic and pathologic events. Development of tumors depends on the supply of nutrients and oxygen from newly formed blood vessels. The process of angiogenesis involves endothelial cell activation, degradation of extracellular matrix (ECM), and basal membrane. This is followed by endothelial cell migration, proliferation, and subsequent differentiation into functional blood vessels (8). Tumor-induced angiogenesis is mediated through the action of some soluble mediators, for instance, vascular endothelial growth factor (VEGF), fibroblast growth factor, angiopoietin-2, and matrix metalloproteinases (MMP; ref. 9). Tumor cells can trigger the release of various proangiogenic factors, such as VEGF and platelet-derived growth factor, where the former initiates the angiogenic process and the latter maintains blood vessels (10).

VEGF mediates its biological effects on the vascular endothelium mainly through the two tyrosine kinase receptors [i.e., VEGF receptor (VEGFR) 1 (Flt-1) and VEGFR-2 (Flk-1; ref. 11)]. The interaction between VEGF-1 and VEGFR-2 is important for angiogenesis during tumor formation. The major

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Requests for reprints: Chi-Hin Cho, Department of Pharmacology, Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Hong Kong, Hong Kong. Phone: 852-2819-9252; Fax: 852-2817-0859. E-mail: chcho@hkusua.hku.hk

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action of VEGF is acting through VEGFR-2 to exert mitogenic and motogenic effects (12). VEGFR-2 tyrosine kinase inhibitors (PTK787 and ZK2284) significantly reduce the kinase activity and thereby inhibit angiogenesis and tumorigenesis in tumor models (13). Other studies have shown that treatment with antibodies against VEGF or VEGFR can suppress angiogenesis (14, 15).

Cancer metastasis proceeds in various steps, such as adhesion to the ECM followed by proteolytic degradation and ultimately migration (16). MMPs are involved in ECM degradation (17) and the activity is associated with invasiveness and metastasis in tumor cells (18). MMP-2 and MMP-9 are the two major enzymes that can selectively degrade type IV collagen and facilitate tumor invasion and metastasis in various experimental models, including gastric, colon, and breast cancers (19-21). Administration of nicotine increased the severity of choroidal neovascularization and also reversed the VEGF-induced suppression of MMP-2 activity in mice (22), showing that MMP could play a role in the angiogenesis induced by nicotine. Indeed, suppression of MMP by different inhibitors markedly reduced tumor cell invasiveness and metastasis (23). However, the direct action of nicotine on angiogenesis and invasion of cancer cells remains unknown, although recent studies show that nicotine can regulate endothelial cell growth (24-26).

In the present study, a sponge implantation model and a cocultured system of vascular endothelial cells and gastric cancer cells were used to study the direct action of nicotine on angiogenesis. A Matrigel invasion chamber was used to investigate the invasiveness of nicotine. The roles of COX-2 and the VEGF/VEGFR system on the actions of nicotine on these pathologic processes were also investigated.

Results

Effect of Nicotine on COX-2 and VEGFR in Gastric Cancer Cell Proliferation

As shown in our previous findings, nicotine (10-200 $\mu\text{g}/\text{mL}$) dose-dependently stimulated gastric cancer cell proliferation through up-regulation of COX-2 expression and increased the release of prostaglandin E_2 and VEGF (4). Both VEGFR-1 and VEGFR-2 were expressed in the unstimulated AGS cells; however, only VEGFR-2 was significantly up-regulated in the nicotine-stimulated cells (Fig. 1A), which was suppressed by SC-236 (selective COX-2 inhibitor) or CBO-P11 (VEGFR inhibitor) pretreatment. These data reveal that both COX-2 and VEGFR-2 are imperative in the action of nicotine on gastric cancer growth.

To further substantiate the role of COX-2 and VEGFR in the nicotine-induced gastric cancer cell proliferation, gastric cancer cells were preincubated with SC-236 (24.8 $\mu\text{mol}/\text{L}$) or CBO-P11 (10 $\mu\text{mol}/\text{L}$) and it was found that they both markedly reduced the stimulatory action of nicotine on cell proliferation by 50% and 37%, respectively (Fig. 1B). In addition, combination of SC-236 and CBO-P11 further suppressed cell proliferation by 70% of the control without the blockers. Nicotine not only exerted mitogenic effect but also promoted the release of proangiogenic factor VEGF by 2.5-fold. This effect was significantly reversed by the COX-2 and VEGFR inhibitors. Combination of SC-236 and CBO-P11 reduced the VEGF content further to the basal control level (Fig. 1C).

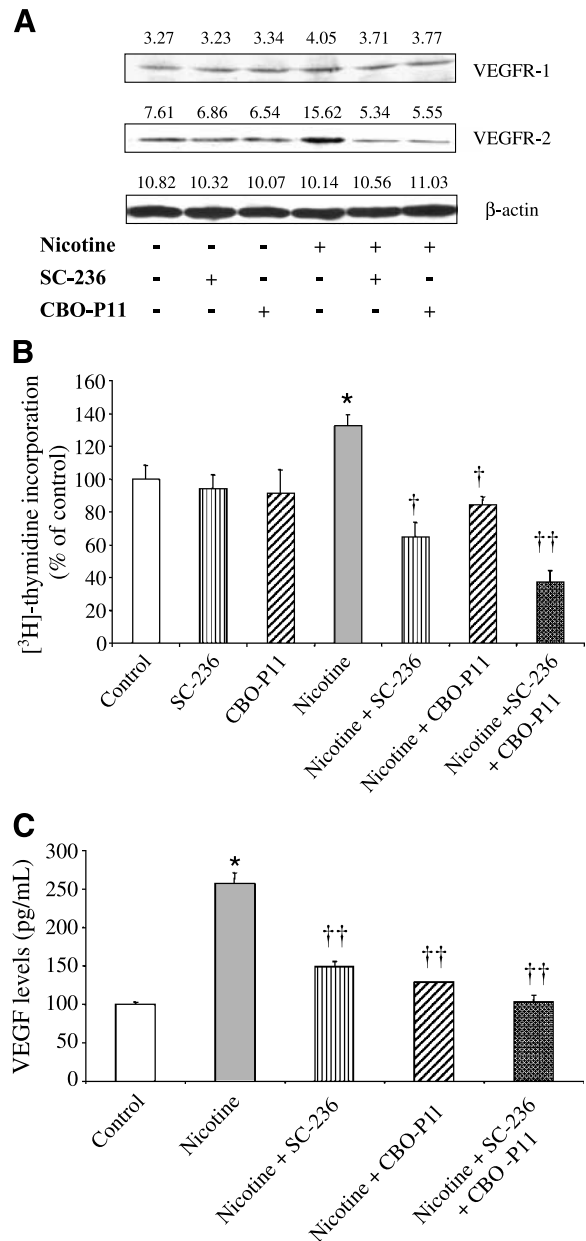


FIGURE 1. A. Western blot analysis of the effect of nicotine on VEGFR-1 and VEGFR-2 protein expressions in AGS cells. Effects of selective COX-2 inhibitor (SC-236, 24.8 $\mu\text{mol}/\text{L}$) or VEGFR inhibitor (CBO-P11, 10 $\mu\text{mol}/\text{L}$) on $[^3\text{H}]$ thymidine incorporation (B) and VEGF levels (C) in the nicotine-mediated gastric cancer cells (AGS). Cells were pretreated with SC-236 or CBO-P11 for 60 minutes before nicotine incubation for 5 hours and the experiments were done as described in Materials and Methods. Columns, mean of two triplicate experiments; bars, SE. *, $P < 0.05$ versus the control group; †, $P < 0.05$; ††, $P < 0.01$ versus the respective nicotine-treated groups.

Involvement of COX-2 and VEGFR in Nicotine-Induced Angiogenesis in a Sponge Implant Model

To examine the direct effect of nicotine on angiogenesis, a sponge implantation model was adopted to study the role of COX-2 and VEGFR in tumor-associated angiogenesis. A schematic diagram of nicotine treatment and inhibitors of COX-2 and VEGFR in the sponge implantation model is shown

in Fig. 2A. The degree of angiogenesis was determined by measuring the hemoglobin content in the sponge implant after 1 month (Fig. 2B). The basic fibroblast growth factor was used as a positive control, which increased the hemoglobin content by ~70% when compared with the control, whereas topical injection of nicotine (20 μ g) significantly enhanced by 26% (Fig. 2C). Administration of the inhibitors for COX-2 (SC-236, 2 mg/kg) and VEGFR (CBO-P11, 0.4 mg/kg) reversed the stimulatory action of nicotine to the control level. Hence, it is implicated that COX-2 and VEGFR are involved in the angiogenesis induced by nicotine.

Levels of VEGF in Sponge Implants

The immunoreactive VEGF protein in the sponge implant was measured with an ELISA kit. Levels of VEGF were significantly increased in both basic fibroblast growth factor- and nicotine-treated groups by 80% and 30%, respectively, when compared with the control (Fig. 2D). Topical injections of SC-236 or CBO-P11 markedly repressed the VEGF content back to the basal level. These results suggest that the induction of VEGF by nicotine is COX-2 and VEGFR dependent and involved in the promotion of angiogenesis in the sponge model.

Effects of COX-2 and VEGFR on Tumor Invasiveness Induced by Nicotine

Invasion is another important process that leads to gastric cancer promotion. To examine the effect of nicotine on tumor invasion, we determined the effect of COX-2 and VEGFR inhibitors on the number of migrated cells invaded through the Matrigel membrane. The invasive ability of tumor cells was increased by nicotine, and the number of invading cells was increased by 4-fold when compared with the control group (Fig. 3). SC-236 and CBO-P11 partially but significantly inhibited the migration of gastric cancer cells through the Matrigel membrane. It is likely that nicotine-induced gastric cancer cell invasion is also COX-2 and VEGFR dependent.

Effect of Nicotine on Endothelial Cells (Human Umbilical Vein Endothelial Cells) Migration Induced by AGS Cells

To validate the roles of COX-2 and VEGFR in the action of nicotine on endothelial cell migration, human umbilical vein endothelial cells were cocultured with tumor cells in the presence of nicotine with or without the respective inhibitor. Nicotine (200 μ g/mL) significantly stimulated endothelial cell migration when cocultured with AGS cells in comparison with the control group. Pretreatment with SC-236 or CBO-P11 before nicotine completely inhibited the stimulatory action of nicotine on endothelial cell migration (Fig. 4). Therefore, it is hypothesized that nicotine could stimulate the release of these mediators to promote endothelial cell migration. However, the mechanism of how and from where nicotine induce COX-2 and VEGF expressions remains unknown.

Effect of Nicotine on MMP-2 and MMP-9 in AGS Cells

In accordance with the stimulatory action of nicotine on the invasiveness of gastric cancer cells, the production of the two major MMPs (MMP-2 and MMP-9) was also measured. Activation of MMPs can selectively degrade type IV collagen,

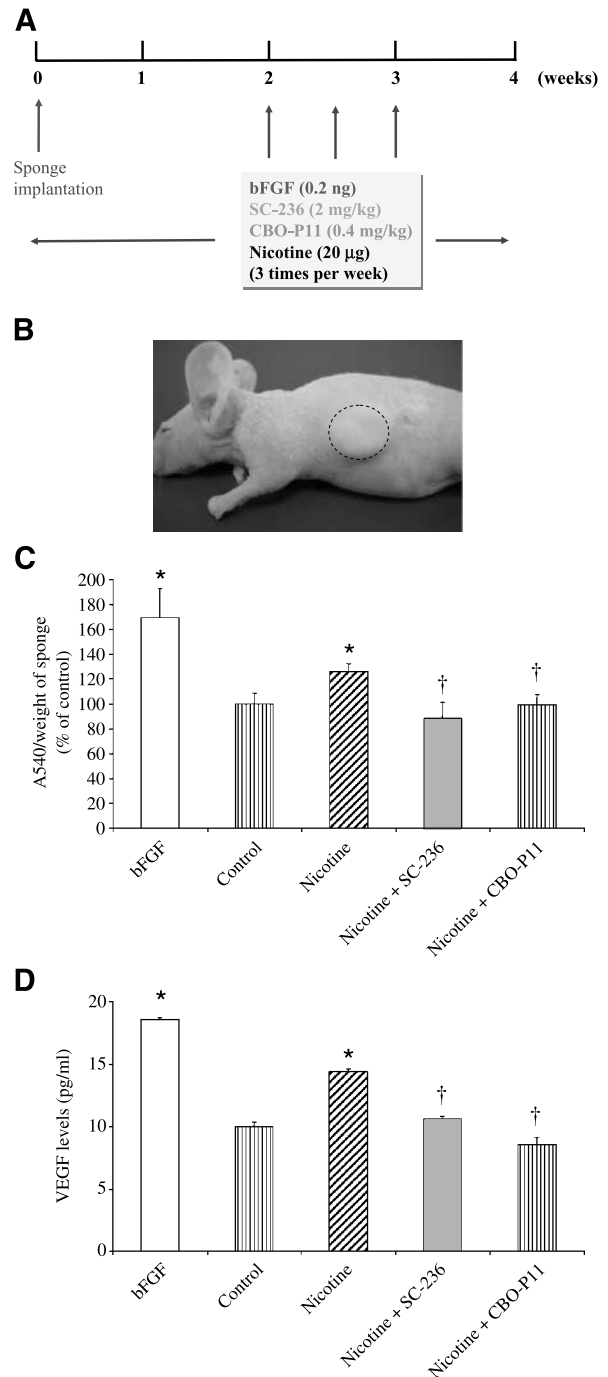


FIGURE 2. **A.** Schematic diagram showing nicotine treatment in the sponge implantation model for 1 month. Basic fibroblast growth factor (bFGF; 0.2 ng), COX-2 inhibitor (SC-236, 2 mg/kg), and VEGFR inhibitor (0.4 mg/kg) were injected topically into the sponge three times weekly throughout the experiment. **B.** Subcutaneous sponge implantation in the dorsal area of nude mice (dotted circle). **C.** Effects of selective COX-2 inhibitor (SC-236, 2 mg/kg) or VEGFR inhibitor (CBO-P11, 0.4 mg/kg) on tumor-associated angiogenesis in the sponge implants. The degree of angiogenesis was determined by measuring the hemoglobin content of the sponge implant as described in Materials and Methods. **D.** Effects of selective COX-2 or VEGFR inhibitor on VEGF levels in the sponge implants. Supernatants from the sponge implants after centrifugation were subjected to VEGF assay. Columns, mean of 10 mice in each group; bars, SE. *, $P < 0.05$ versus the control group; †, $P < 0.05$ versus the respective nicotine-treated groups.

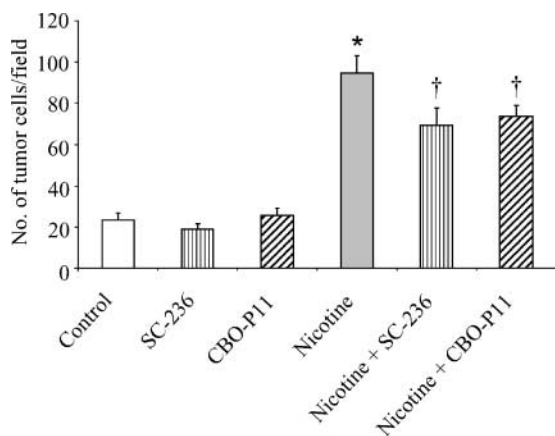


FIGURE 3. Effects of selective COX-2 inhibitor (SC-236, 24.8 $\mu\text{mol/L}$) or VEGFR inhibitor (CBO-P11, 10 $\mu\text{mol/L}$) on the invasion ability of nicotine-stimulated gastric cancer cells (AGS). The experiments were done as described in Materials and Methods. Numbers of AGS cells migrated through the Matrigel layer to the lower surface of the filter were counted under light microscope ($\times 200$). Columns, mean of two triplicate experiments; bars, SE. *, $P < 0.05$ versus the control group; †, $P < 0.05$ versus the respective nicotine-treated groups.

which attributes to tumor invasion and metastasis. Nicotine significantly increased MMP-2 (72 kDa) and MMP-9 (92 kDa) activities and protein expressions (Figs. 5 and 6). Nicotine increased the activities of MMP-2 (Fig. 5) and MMP-9 (Fig. 6) by 70% and 40%, respectively, when compared with the control group. On the other hand, pretreatment with SC-236 or CBO-P11 markedly reduced the activities and protein expressions of MMP-2 and MMP-9, suggesting that the action of nicotine on these proteins was also COX-2 and VEGFR dependent.

Effect of Nicotine on Urokinase-Type Plasminogen Activator and Its Receptor and Plasminogen Activator Inhibitor-1 in AGS Cells

Plasminogen activators are enzymes that degrade proteins in tissue basement membranes and ECM, which is implicated in neovascularization, invasion, and metastasis of solid tumors. Nicotine up-regulated the urokinase-type plasminogen activator (uPA) and its receptor (uPAR) expression; however, it did not affect the expression of plasminogen activator inhibitor-1 (Fig. 7). Treatment with SC-236 or CBO-P11 reversed the stimulatory action of nicotine on uPA and uPAR expression back to the basal level. These results reveal that COX-2 and VEGFR are involved in the excitatory action of nicotine on uPA system leading to the increase of invasion and angiogenesis during tumorigenesis of gastric cancers.

Discussion

Ample evidences both *in vivo* and *in vitro* have reported that COX-2 is closely associated with carcinogenesis. Both selective and nonselective COX inhibitors were shown to effectively abrogate the progression of tumors in animal models (6, 27). Nicotine has been shown to induce hypothalamic-pituitary-adrenal activity, which is related to prostaglandin synthesis (28, 29). Up-regulation of COX-2 mRNA expression was observed after exposure to nicotine in human gingival

fibroblasts and rat microglial cells (30, 31). All these provide evidence that nicotine, the active component in cigarette smoke, could play a role in the pathogenesis of smoking-associated disease, which is likely to be COX/prostaglandin dependent. In the present study, selective COX-2 inhibitor (SC-236) markedly reduced nicotine-induced gastric cancer cell proliferation and VEGF levels (Fig. 1B). Indeed, the antitumor action of COX-2 inhibitors is suggested to be due to its induction of apoptosis and suppression of cell proliferation. However, experimental data reporting the ability of COX-2 and its relationship with VEGF on angiogenesis and metastasis, the two important processes in tumor development, are limited.

Nicotine has been shown to increase the vascularity of choroidal neovascularization through the induction of VEGF (22). Overexpression of VEGFR is found in angiogenic blood vessels in various human cancers (32, 33). Recent study shows that VEGFR plays a significant role in the development of gastric cancer and also in lymph node metastasis (34). To ascertain the role of COX-2 and VEGFR in the action of nicotine on tumor-associated angiogenesis, we adopted a sponge implantation model. In this model, nicotine increased tumor-associated angiogenesis by $\sim 26\%$ when compared with the control group (Fig. 2C). Topical injection of the inhibitors of COX-2 and VEGFR significantly reduced the hemoglobin content in sponge implants, implicating that the promoting action of nicotine on angiogenesis is COX-2 and VEGFR dependent. In addition, nicotine also stimulated the release of VEGF from adjacent tissues into sponge implants, which was again blocked by inhibitors of COX-2 and VEGFR (Fig. 2D). Both VEGFR-1 and VEGFR-2 proteins were expressed in gastric cancer cells, but only VEGFR-2 expression was significantly up-regulated by nicotine and blocked by SC-236 and CBO-P11 (Fig. 1B). Our previous study also showed that similar concentration of nicotine stimulated the secretion of VEGF from gastric cancer cells (4). It is likely that the

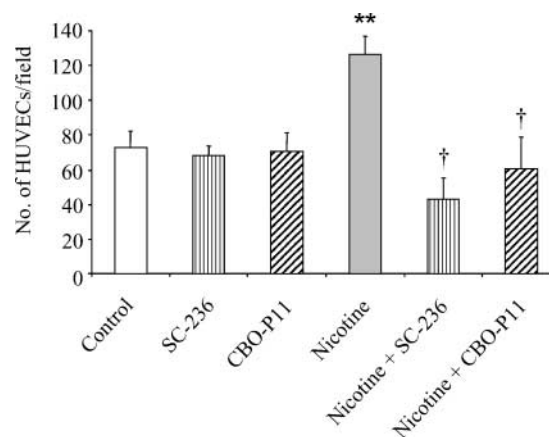


FIGURE 4. Effects of selective COX-2 inhibitor (SC-236, 24.8 $\mu\text{mol/L}$) or VEGFR inhibitor (CBO-P11, 10 $\mu\text{mol/L}$) on migration of cocultured human umbilical vein endothelial cells (HUVECs). The experiments were done as described in Materials and Methods. Cell movement was evaluated by the number of cells that had migrated to the conditioned medium of tumor cells with different treatments and counted under light microscope ($\times 200$). Columns, mean of two triplicate experiments; bars, SE. **, $P < 0.01$ versus the control group; †, $P < 0.05$ versus the respective nicotine-treated groups.

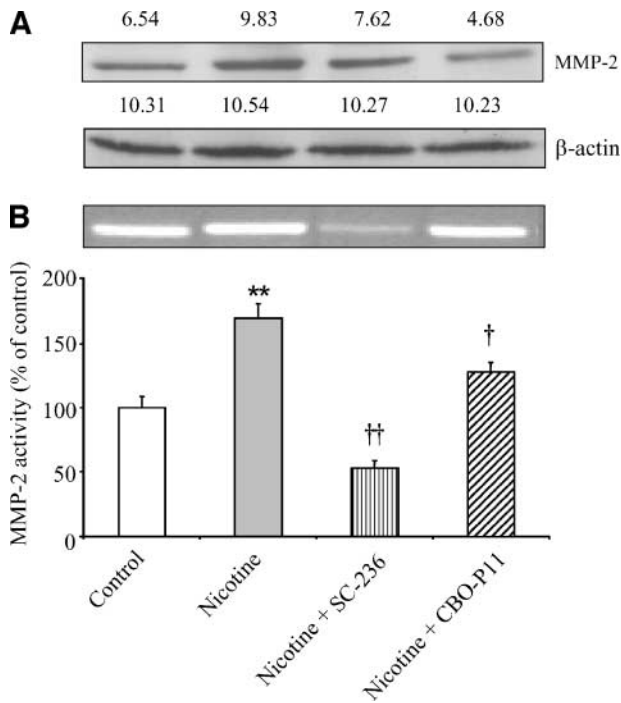


FIGURE 5. Effects of selective COX-2 inhibitor (SC-236, 24.8 $\mu\text{mol/L}$) or VEGFR inhibitor (CBO-P11, 10 $\mu\text{mol/L}$) on MMP-2 expression and activity. **A.** Western blot analysis of MMP-2 protein. **B.** Zymography of MMP-2 activity. The experiments were done as described in Materials and Methods. Columns, mean of two triplicate experiments; bars, SE. **, $P < 0.01$ versus the control group; †, $P < 0.05$; ††, $P < 0.01$ versus the respective nicotine-treated groups.

mitogenic effect induced by nicotine depends on the activation of COX-2 with the release of VEGF that binds to the VEGFR, particularly VEGFR-2, in gastric cancer cells. It was interesting to note that blocking the VEGFR by CBO-P11 abrogated the stimulatory action of nicotine on VEGF level in the sponge (Fig. 2D) as well as in gastric cancer cells (Fig. 1C). These findings suggest that VEGFRs could have a positive feedback action on the release of VEGF from normal and cancer cells. Corresponding study also indicated that injection of VEGFR inhibitor reduced the release VEGF from tumors in mice (35). Furthermore, the intimate relation between VEGF and COX-2/prostaglandin was also shown in other gastric cell line (MKN-28 cells) and gallbladder carcinoma (36, 37).

To further understand the action of nicotine in tumor angiogenesis and invasion, the Matrigel invasion chamber was used to study the invasiveness of AGS cells stimulated by nicotine. Results showed that nicotine stimulated the invasive ability of gastric cancer cells, and inhibitors of COX-2 and VEGFR markedly prevented cancer cell migration from the upper chamber to the lower chamber (Fig. 3). Furthermore, nicotine was able to promote angiogenesis through endothelial cell migration in the coculture system of human umbilical vein endothelial cells and tumor cells (AGS cells). This was again attenuated by SC-236 and CBO-P11 (Fig. 4). These results suggest that COX-2 produced by AGS cells could stimulate the release of some growth factors, which is likely to be VEGF to enhance the migration of endothelial cells. In concord with the

current finding, earlier study also reported that COX-2-expressing tumor cells stimulated the mobility of cocultured endothelial cells with the induction of VEGF (38). Therefore, it is suggested that the stimulatory action of nicotine on invasiveness (migration of cancer cells) and angiogenesis (mobility of endothelial cells) are dependent on COX-2 activity and the angiogenic action of VEGF. This phenomenon is in accord with other study that inhibitor of COX-2 markedly reduces *Helicobacter pylori*-promoted gastric cancer cell invasion, concomitant with down-regulation of MMP-9 and VEGF protein expressions (39).

Because COX-2 has been localized in tumor cells and adjacent stroma cells, the release of prostaglandins from COX-2 may act on the tumor cells or the neighboring stroma cells to facilitate the development of tumors (40, 41). To further enhance the invasiveness of cancer cells, degradation of ECM would facilitate the growth and metastasis of tumors. MMP-2, MMP-9, and uPAs (uPA and uPAR) are important proteolytic enzymes for the degradation of ECM. MMP-2 and MMP-9 activities as well as their protein expressions were markedly increased in the nicotine-treated groups (Figs. 5 and 6). Furthermore, expressions of uPA and uPAR were strongly induced by nicotine. In contrast, the induction of these proteolytic enzymes was suppressed by SC-236 and CBO-P11 (Figs. 5-7). Phorbol 12-myristate 13-acetate, a tumor-promoting agent, also induces tumor cell invasion through the induction of MMP-9 in AGS cells (19). There have been several reports

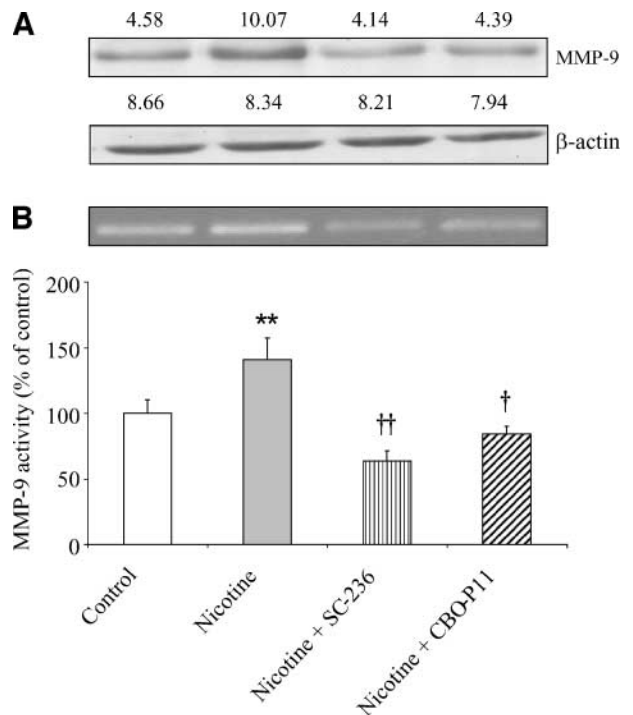


FIGURE 6. Effects of selective COX-2 inhibitor (SC-236, 24.8 $\mu\text{mol/L}$) or VEGFR inhibitor (CBO-P11, 10 $\mu\text{mol/L}$) on MMP-9 expression and activity. **A.** Western blot analysis of MMP-9 protein. **B.** Zymography of MMP-9 activity. The experiments were done as described in Materials and Methods. Columns, mean of two triplicate experiments; bars, SE. **, $P < 0.01$ versus the control group; †, $P < 0.05$; ††, $P < 0.01$ versus the respective nicotine-treated groups.

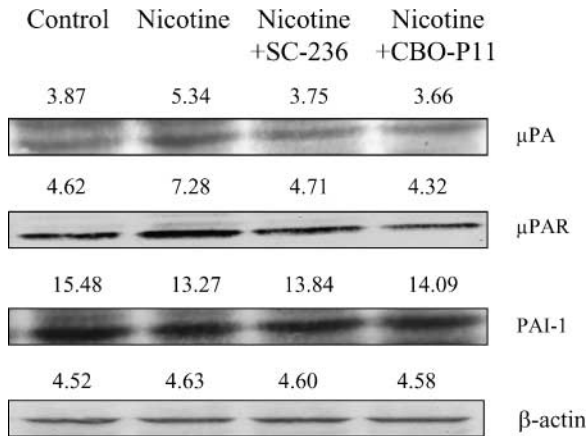


FIGURE 7. Western blot analysis of the effects of selective COX-2 inhibitor (SC-236, 24.8 μ mol/L) or VEGFR inhibitor (CBO-P11, 10 μ mol/L) on uPA, uPAR, and plasminogen activator inhibitor-1 (PAI-1) protein expressions. The experiments were done as described in Materials and Methods. Western blot data were obtained in three independent experiments.

showing that other MMPs also facilitate tumor invasion and metastasis and are related to the initial step of gastric cancer invasion (42, 43). Elevated levels of uPA and plasminogen activator inhibitor-1 in cancer tissues are independent prognostic indicators of poor survival in gastric cancer patients (44). uPA is associated with tumor cells or malignant transformation, whereas uPAR plays a pivotal role in generating the proteolytic cascade to unleash MMPs for basement membrane remodeling by tumor cells and endothelial cells (45).

Nonsteroidal anti-inflammatory drugs have been reported to reduce the risk of various cancers by inhibiting COX activity. This can reduce tumor cell proliferation and invasion. It has also been shown that COX-2 inhibitor significantly reduced tumor-induced MMP-2 and MMP-9 expression in cancer models (21, 46). To elucidate the possible link between COX-2/VEGFR and the proteolytic enzymes, cells were treated with SC-236 or CBO-P11 to examine the effects on MMP-2 and MMP-9 as well as uPA and uPAR on gastric cancer cells. It was found that inhibitors of COX-2 and VEGFR significantly suppressed the activation of nicotine on MMP-2 and MMP-9 activities and also uPA and uPAR expression (Figs. 5-7). These data support the notion that the action of nicotine on cancer cell invasion and tumor angiogenesis is via the activation of MMPs and uPAs, which are COX-2 and VEGFR dependent. It has been reported that VEGF can increase the binding of uPA to uPAR and enhance the proteolytic activity of invading cells by stimulating the release of MMPs (47). However, the relationship between uPA and MMP activation in the signaling pathway stimulated by nicotine to promote invasion and angiogenesis of gastric cancer cells needs further study.

In conclusion, nicotine can stimulate COX-2 expression to trigger tumor cell invasion and angiogenesis through activation of VEGF, which subsequently modulates the MMP activity and expression of plasminogen activators. Inhibitors of COX-2 and VEGFR suppress this biological pathway resulting in the

inhibition of tumor cell invasion and angiogenesis (Fig. 8). Hence, it is likely that agent that can block the COX-2/VEGF pathway is effective in the suppression of tumor invasion and angiogenesis in gastric cancer.

Materials and Methods

Reagents and Drugs

SC-236 (4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzenesulfonamide) was purchased from Pharmacia (Peapack, NJ). Antibodies specific to COX-2, VEGFR-1, and VEGFR-2 were from Santa Cruz Biotechnology (Santa Cruz, CA). CBO-P11 (VEGFR inhibitor) was purchased from Calbiochem (Darmstadt, Germany). Nicotine was from Sigma Chemical Co. (St. Louis, MO). VEGF enzyme immunoassay kit was obtained from Oncogene (Darmstadt, Germany). Other chemicals and reagents were from commercial sources unless otherwise specified.

Animals

This study was approved by the Committee on the Use of Live Animals in Teaching and Research at the University of Hong Kong. Athymic BALB/c *nu/nu* mice supplied by Charles River Laboratories (Wilmington, MA), 4 to 6 weeks old, were fed with a standard laboratory diet (Ralston Purina Co., Chicago, IL) *ad libitum*. They were housed in individually ventilated cages with isolated ventilation in a holding room controlled at $22 \pm 1^\circ\text{C}$ and 65% to 70% relative humidity with a 12:12-hour light/dark cycle.

Sponge Implantation Model of Angiogenesis

The sponge implantation model was developed to study angiogenesis *in vivo* as described previously with some modifications (48). Sponge discs (diameter, 0.7 cm; thickness,

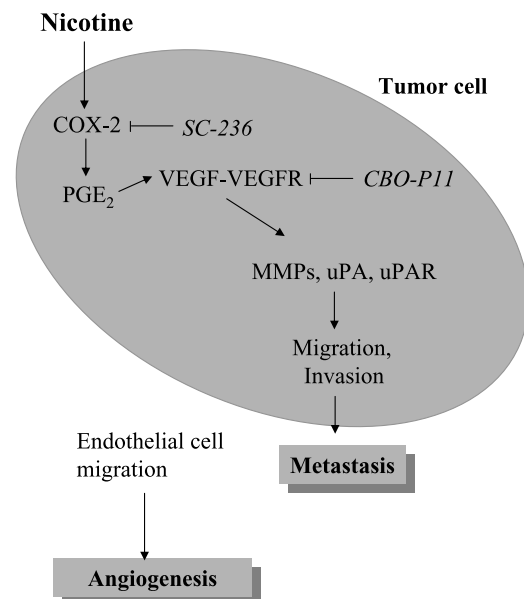


FIGURE 8. Proposed mechanisms of the regulatory roles of COX-2 and VEGFR in tumor cell invasion and metastasis. PGE_2 , prostaglandin E_2 .

0.2 cm) were prepared from the polyester polyurethane foam. The discs were disinfected by soaking in 70% ethanol for 3 hours and then rinsed in distilled water and finally sterilized with UV irradiation overnight. Sterilized sponge disc was implanted subcutaneously in the dorsal area of nude mice (Fig. 2B). After implantation, they were then divided into different groups: (a) basic fibroblast growth factor (0.2 ng) positive control; (b) control; (c) nicotine (20 μ g); (d) nicotine (20 μ g) + SC-236 (2 mg/kg); and (e) nicotine (20 μ g) + CBO-P11 (0.4 mg/kg). Mice were sacrificed after 1 month and the sponges were taken out for measuring hemoglobin content and VEGF levels.

Cell Culture and Drug Treatment

Human gastric adenocarcinoma cells (AGS) were purchased from American Type Culture Collection (Manassas, VA) (CRL-1739). Cells were cultured in RPMI 1640 (Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (Life Technologies), 100 units/mL penicillin G, and 100 μ g/mL streptomycin. Human umbilical vein endothelial cells were purchased from American Type Culture Collection (CRL-1730). Cells were maintained in Kaighn's F12K medium (F12K) containing 20% fetal bovine serum, 1% antibiotic-antimycotic, and 75 μ g/mL endothelial cell growth supplement. Cells were maintained at 37°C, 95% humidity, and 5% CO₂.

AGS cells were plated at a density of 8×10^4 per well in 24-well plates. At confluence, nicotine (200 μ g/mL) was incubated with cells for 5 hours to study the mitogenic effect of nicotine on gastric cancer cells. To examine the effects of various inhibitors, cells were pretreated with or without SC-236 (24.8 μ mol/L for 60 minutes) or CBO-P11 (10 μ mol/L for 60 minutes) before nicotine treatment (200 μ g/mL for 5 hours). Control cells were allowed to grow in the absence of SC-236 or any inhibitors for the same period of time as the treated cells. Experiments were done in duplicate and repeated three times. The concentration of nicotine used in the present study mimicked the daily intakes of cigarettes in heavy smokers (>30 cigarettes per day; ref. 49).

[³H]Thymidine Incorporation Assay

A modified [³H]thymidine incorporation assay was used to determine the amount of DNA synthesis (50). After confluent, cells were incubated in the presence or absence of SC-236 (24.8 μ mol/L for 60 minutes) or CBO-P11 (10 μ mol/L for 60 minutes) before nicotine (200 μ g/mL for 5 hours) and then incubated with 0.5 μ Ci/mL [³H]thymidine (Amersham Corp., Arlington Heights, IL) for 5 hours. It was then washed with ice-cold 0.15 mol/L NaCl followed by 10% trichloroacetic acid and incubated for 15 minutes at room temperature. After several washing, 1% SDS was added and incubated for another 15 minutes at 37°C. Finally, hydrophilic scintillation fluid was added into the vial and the amount of DNA synthesized was measured using a liquid scintillation counter coupled to a β -counter (Beckman Instruments, Fullerton, CA).

Immunoblotting

Whole-cell lysates were prepared as described previously (51). Briefly, cells were lysed in 500 μ L radioimmunoprecipitation

assay buffer and proteinase inhibitors. Protein was quantified with the use of protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (80 μ g/lane) were resolved with SDS-PAGE and transferred to Hybond C nitrocellulose membranes (Amersham). The membranes were probed with COX-2, VEGFR-1, or VEGFR-2 antibodies overnight at 4°C and incubated for 1 hour with secondary peroxidase-conjugated antibody. The membranes were developed with an enhanced chemiluminescence system (Amersham) and exposed to an X-ray film (Fuji Photo Film Co. Ltd., Tokyo, Japan). Quantitation was carried out with a video densitometer (ScanMaker III, Microtek).

Hemoglobin Assay

Neovascularization is determined in terms of hemoglobin concentration. Hemoglobin content was assessed with the use of Drabkin's reagent as described previously (52). Sponges were homogenized in 50 μ L ultrapure water using a pestle and incubated with 0.5 mL Drabkin's reagent for 15 minutes at room temperature. Homogenates were then centrifuged at $1,000 \times g$ for 10 minutes and the absorbance of the supernatant was measured at 540 nm. The total hemoglobin concentration was expressed as the absorption divided by the weight of the sponge.

Invasion Assay

The invasive ability of cells was assessed by using BioCoat Matrigel Invasion Chamber as described previously (53). Briefly, tumor cells were treated with COX-2 and VEGF inhibitors. After 5 hours of drug treatment, cells were trypsinized and suspended in phenol red-free culture medium in the absence of serum at a concentration of 1×10^6 /mL in the upper chamber. The lower chamber was filled with culture medium supplemented with 10% FCS as the chemoattractant (Fig. 3). After 24 hours, invaded cells that had passed through the Matrigel and adhered to the lower surface of the membrane were fixed with methanol, stained with 0.1% crystal violet, and counted under a light microscope ($\times 200$).

Migration of Endothelial Cells In vitro

In vitro indirect coculture assay was done to measure the migration of endothelial cells during angiogenesis through Falcon cell culture insert with 0.4- μ m pore size PET membrane (54). Gastric cancer cells were grown to confluence in the lower chamber and incubated with nicotine in the absence or presence of inhibitors of COX-2 and VEGFR for 5 hours. Human umbilical vein endothelial cells were then suspended in serum-free medium in the upper chamber. The cocultured cells were incubated for another 5 hours and the number of migrated endothelial cells was counted under a light microscope ($\times 200$).

Gelatin Zymography

Gelatinolytic enzymes in AGS cells were analyzed by zymography as described previously (55). In brief, cells were incubated with nicotine in the absence or presence of SC-236 pretreatment. The supernatants were collected after 5 hours and centrifuged at $17,689 \times g$ for 10 minutes. The supernatants were resolved in SDS sample buffer without mercaptoethanol

and electrophoresed in 7.5% polyacrylamide gel containing 1 mg/mL gelatin. The gel was washed with 2.5% Triton X-100 to remove SDS and incubated with developing buffer (containing 50 mmol/L Tris-HCl, 0.2 mol/L NaCl, 5 mmol/L CaCl₂, 0.02% Brij-35) overnight at 37°C. To visualize the presence of gelatinolytic bands, gels were stained with Coomassie blue (R-250) and destained with Coomassie blue destaining solution (R-250) until all bands of lysis became clear.

VEGF Assay

Supernatants were collected from the sponge implants and the levels of VEGF were analyzed using a VEGF ELISA kit (Oncogene Research Products, EMD Biosciences, Inc., Darmstadt, Germany). This detection method was based on a competitive reaction between biotinylated VEGF conjugate and samples for specific VEGF antibody binding sites, giving an inverse relationship between absorbance and VEGF concentrations, which were expressed as pg/mL.

Cell Viability

Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction method (56). After incubation with nicotine at 200 µg/mL for 5 hours, cells were incubated with 2.5% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (5 mg/mL) for another 4 hours at 37°C. Thereafter, 0.04 mol/L HCl-isopropanol was added into the mixture and mixed thoroughly. The color change was recorded using spectrophotometry with the microplate reader (MRX, Dynex Technologies, Inc., Chantilly, VA) at 570 nm. The same test was repeated three times and the absorbance was calculated for statistical analysis.

Statistical Analysis

Results were expressed as mean ± SE. Statistical analysis was done using ANOVA followed by the Tukey *t* test. *P*s < 0.05 were considered statistically significant.

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