Nicotine promotes gastric tumor growth and neovascularization by activating extracellular signal-regulated kinase and cyclooxygenase-2

Vivian Y. Shin1, William K.K. Wu1, Yi-Ni Ye1, Wallace H.L. So1, Marcel W.L. Koo1, Edgar S.L. Liu1, Jiing-Chyuan Luo2 and Chi-Hin Cho1,3

1Department of Pharmacology, Faculty of Medicine, The University of Hong Kong, Hong Kong, HKSAR, China and 2Division of Gastroenterology, Department of Medicine, Taipei Veterans General Hospital and National Yang-Ming University, School of Medicine, Taipei, Taiwan

Email: chcho@hkusua.hku.hk

Early studies revealed that cigarette smoke promotes gastric cancer growth through the induction of cyclooxygenase-2 (COX-2). Nicotine, one of the active ingredients in cigarette smoke, has detrimental effects in the stomach. To date, there is no direct evidence to validate the effect of nicotine on gastric tumor growth and its carcinogenic mechanism(s). We therefore investigated whether nicotine could promote tumor growth and neovascularization in vivo, and the biological mechanism(s) in connection with the signaling cascade involving COX-2 and extracellular signal-regulated protein kinase (ERK). Athymic nude mice, with gastric cancer cells (AGS) orthotopically implanted into the gastric wall, treated with nicotine (50 or 200 µg/ml) in their drinking water for 3 months developed larger tumor areas than mice in the control group. Nicotine further increased proliferating cellular nuclear antigen (PCNA) staining and microvessel density by 70 and 30%, respectively, with concomitant activation of ERK phosphorylation, COX-2 and vascular endothelial growth factor (VEGF) expression in the tumors. Intraperitoneal administration of a selective COX-2 inhibitor (SC-236, 2 mg/kg) prevented the nicotine-induced tumor growth and neovascularization dose-dependently. Consistent with our animal model, an in vitro study also demonstrated that incubation with nicotine (50–200 µg/ml) for 5 h stimulated cell proliferation dose-dependently and increased COX-2 expression, prostaglandin E2 (PGE2) and VEGF release, as well as activation of ERK phosphorylation. Pre-treatment with specific mitogen-activated protein kinase kinase (MEK) inhibitors (U0126 or PD98059) attenuated COX-2 expression and subsequent PGE2 release by nicotine. Furthermore, the stimulatory action of nicotine on cancer cell growth and angiogenic factor VEGF production was suppressed by inhibitors of MEK (U0126) and COX-2 (SC-236). These findings reveal a direct promoting action of nicotine on the growth of gastric tumor and neovascularization through sequential activation of the ERK/COX-2/VEGF signaling pathway, which can be targeted for chemoprevention of gastric cancer, particularly in cigarette smokers.

Introduction

Cigarette smoking is a major public health issue, and is closely associated with gastric cancer. Considerable evidence demonstrates that smokers have a higher risk for developing various types of cancers than non-smokers (1–3). There are more than 4500 compounds in cigarette smoke, making it difficult to study their adverse action in biological systems. Recent research has focused on the deleterious effects of cigarette smoke, particularly nicotine on the stomach. As nicotine is currently used for replacement therapy on smoking cessation in different forms from skin patches to the oral route in the form of gum or lozenges, numerous studies investigated the effects of nicotine on the stomach, but results have been equivocal. Previous reports demonstrated that chronic nicotine administration worsens ethanol-induced ulceration, while acute nicotine treatment protects against ethanol ulceration in rats (4). Moreover, nicotine treatment and its withdrawal potentiated stress-induced gastric ulceration (5). Other investigators indicated that nicotine enhances chemical-induced gastric carcinogenesis (6,7). However, there is limited evidence for the direct action of nicotine on gastric tumor growth, and furthermore, the carcinogenic mechanism(s) has not been defined.

Extensive use of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) reduces the incidence of gastric cancer (8) and other gastrointestinal cancers (9). NSAIDs inhibit cyclooxygenases (COXs), which are important enzymes for prostaglandin (PG) biosynthesis. Amongst the two COX isofoms (COX-1 and COX-2), COX-2 is not only a marker of inflammation, but has also been reported to have significant relevance to carcinogenesis in the stomach (10,11). COX-2 is over-expressed in most malignant tissues but barely expressed in normal tissues (12). It is implicated in differentiation and inhibition of programmed cell death in gastric cells (13). Nicotine and its derivatives actively stimulate prostaglandin E2 (PGE2) release in whole blood and macrophages, thus providing an insight into the mechanism of action of nicotine in tumorigenesis (14,15). The relationship between nicotine and COX-2 expression has not been established in the stomach. Therefore, it is essential to examine the effect of nicotine on this promising target involved in gastric carcinogenesis.

Extracellular signal-regulated kinases (ERK-1/2) belong to the mitogen-activated protein kinase (MAPK) family and are crucial in the control of cell growth, cell differentiation and cell survival. On stimulation, they rapidly translocate into the nucleus and trigger the downstream mediator for cell growth. Over-expression of ERK is found in most gastric tumors and is involved in the carcinogenesis of gastric cancer. Thus, ERK is
thought to be one of the possible signaling pathways involved in nicotine-induced cell proliferation. ERK-1/2 are activated by phosphorylation of tyrosine and threonine residues. Nicotine exerts its biological effect through binding to nicotinic receptors, resulting in phosphorylation of ERK and stimulation of DNA synthesis in pulmonary neuroendocrine cells (16). Furthermore, nicotine induces Ca
$^{2+}$ influx and stimulates the Ras/ERK cascade that promotes cell survival in neuronal cells (17). A large body of evidence has demonstrated that ERK is an essential mediator for cell survival, however, its role in relation to nicotine-enhanced carcinogenesis in the stomach remains to be elucidated.

In view of these observations, the present study was designed to evaluate the carcinogenic action of nicotine in the stomach, and further to examine whether ERK and COX-2 are responsible for tumor growth and tumor-associated angiogenic vascular endothelial growth factor (VEGF) expression both in vivo and in vitro. The results of this study validate the carcinogenic action of cigarette smoking in gastric cancer and reveal a potential therapeutic target for gastric cancer prevention in smokers.

**Materials and methods**

**Reagents and drugs**

4-[5-(4-Chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide (SC-236) was purchased from Pharmacia (Peapack, NJ), PD98059 [specific mitogen-activated protein kinase kinase (MEK-1) inhibitor] and U0126 (specific MEK-1/2 inhibitor) were from Cell Signalling Technology (Beverly, MA). Antibodies to COX-1, COX-2, VEGF, p-ERK and cytosolic phospholipase A$_2$ (cPLA$_2$) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Other chemicals and reagents were from Sigma (St Louis, MO) unless otherwise specified.

**Animals**

This study was approved by the Committee on the Use of Live Animals in the Teaching and Research at the University of Hong Kong, Athymic BALB/c-nu/nu mice, 4-6 weeks old, were fed with a standard laboratory diet (Ralston Purina, Chicago, IL) ad libitum. They were housed in IVF cages with isolated ventilation in a holding room controlled at 22 ± 1°C and 65-70% relative humidity with a 12/12 h light/dark cycle.

**Tumor implantation and experimental protocol**

Mice were implanted with AGS (a poorly differentiated human gastric adenocarcinoma cell line), as a human gastric cancer xenograft model (18) for the study of the biological actions of nicotine. AGS cells were trypsinized, and the total cell number in the cell suspension was adjusted to 2 × 10$^7$ cells/ml. A volume of 100 $\mu$l of this cell suspension was inoculated into the gastric walls of mice. Mice were randomly divided into 10 groups: normal control (no AGS inoculation), tumor control, SC-236 (1 mg/kg), SC-236 (2 mg/kg), Nic (50 $\mu$g/ml), Nic (50 $\mu$g/ml) + SC-236 (1 mg/kg), Nic (50 $\mu$g/ml) + SC-236 (2 mg/kg), Nic (200 $\mu$g/ml), Nic (200 $\mu$g/ml) + SC-236 (1 mg/kg), Nic (200 $\mu$g/ml) + SC-236 (2 mg/kg).

The mice received nicotine solution at a dose of 50 or 200 $\mu$g/ml or the control (tap water) for 3 months after tumor implantation. The selective COX-2 inhibitor (SC-236, 1 or 2 mg/kg) was injected intraperitoneally three times a week throughout the experiment. Mice were killed after 3 months of nicotine administration, and the tumors growing on the stomach were removed. The cancerous area (in mm$^3$) was measured and examined histologically.

**Cell culture and drug treatment**

Human gastric adenocarcinoma cells (AGS) were purchased from the American Type Culture Collection (CRL-1739, ATCC, USA). Cells were cultured in RPMI 1640 (GibcoBRL, Grand Island, USA) containing 10% fetal bovine serum (GibcoBRL), 100 U/ml penicillin G, 100 $\mu$g/ml streptomycin, and maintained at 37°C, 95% humidity and 5% carbon dioxide.

AGS cells were plated at a density of 8 × 10$^4$ cells/well in 24-well plates. At confluence, various concentrations of nicotine (10–200 $\mu$g/ml) were incubated with the cells for 5 h to study the mitogenic effect of nicotine on gastric cancer cells. In order to examine the effects of various inhibitors, cells were treated with or without SC-236 (10 $\mu$g/ml for 60 min), PD98059 (20 $\mu$g/ml for 60 min) or U0126 (20 $\mu$g/ml for 60 min) prior to nicotine treatment (50–200 $\mu$g/ml for 5 h). 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 performed in duplicate and repeated three times. The concentrations of nicotine used in the present study mimicked the daily intakes of cigarettes in smokers (light, <10-15 cigarettes/day; moderate, 16-30 cigarettes/day; heavy, > 30 cigarettes/day) (19).

**Immunohistochemistry**

The tumor samples were fixed in formalin overnight and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (H&E) for histopathological assessment of neoplastic lesions. Cell proliferation was assessed by immunostaining as described previously (20) with modifications. Sections were digested with trypsin for 15 min at room temperature and incubated with a blocking agent (LSAB kit, DAKO, Copenhagen, Denmark) for 1 h. They were then incubated with a monoclonal primary antibody against mouse proliferating cell nuclear antigen (PCNA, 1:200) (Santa Cruz Biotechnology, Santa Cruz) overnight at 4°C. The sections were incubated with Link reagent (LSAB kit) for 1 h, followed by streptavidin for another 1 h. Finally they were incubated with hydroxy-peroxide-diaminobenzidine to visualize the PCNA-positive cells. After washing with tap water, the sections were counterstained with Mayer’s hematoxylin, dehydrated and mounted. The number of immunostained tumor cells was expressed as the number of PCNA-positive cells in five to eight randomly selected fields ($\times$200).

**Microvessel density**

Microvessel density was measured with a procedure similar to PCNA-staining, except that a rabbit anti-human von Willebrand factor (1:200) (DAKO) was used to identify microvessels, and Mayer’s hematoxylin counterstaining after hydroxy-peroxide-diaminobenzidine incubation was omitted. Microvessel density was expressed as the number of microvessels per mm$^2$ in five to eight randomly selected fields ($\times$200).

**PGE$\_2$ assay**

Gaistic tissues were homogenized in enzyme immunoassay buffer (containing 50 mM Tris–HCl at pH 7.4, 100 mM NaCl, 1 mM CaCl$_2$, 1 mg/ml p-glucose and 29 $\mu$m indomethacin) for 30 s on ice. Samples were then centrifuged for 15 min at 10000 r.p.m. at 4°C. The supernatant was used for the determination of PGE$\_2$ using a PGE$\_2$ immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**VEGF assay**

Culture media were collected from cells treated with or without nicotine for 5 h, and the levels of VEGF were analyzed using a VEGF ELISA kit (Oncogene Research Products, EMD Biosciences, Darmstadt, Germany). This detection method is based on a competitive reaction between biotinylated VEGF conjugate and samples for specific VEGF antibody binding sites, giving an inverse relationship between optical density (OD) and VEGF concentration, which was expressed as pg/ml.

**$[^{3}H]$Thymidine incorporation assay**

A modified $[^{3}H]$thymidine incorporation assay was used to determine the amount of DNA synthesis (21). Cells were left untreated or were incubated with nicotine or absence of nicotine (10, 50, 100 or 200 $\mu$g/ml) for 5 h and then incubated with 0.5 $\mu$Ci/ml $[^{3}H]$thymidine (Amersham, Arlington Heights, IL) for 5 h. After washing with ice-cold 0.15 M NaCl, followed by 10% trichloroacetic acid, the cells were incubated for 15 min at room temperature. After several washes, 1% sodium dodecyl sulfate (SDS) was added and incubation continued for another 15 min at 37°C. Finally, hydrophilic scintillation fluid was added to the vial and the amount of DNA synthesized was measured by liquid scintillation spectrometry with a beta-counter (Beckman Instruments, Fullerton, CA).

**Immunoblotting**

Whole cell lystate were prepared as described previously (22). Briefly, cells were lysed in 50 $\mu$l of RIPA buffer and proteinase inhibitors. Protein was quantified with a protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (80 $\mu$g/lane) were resolved with SDS-polyacrylamide gel electrophoresis, and transferred to Hybond C nitrocellulose membranes (Amersham). The membranes were probed with COX-1, COX-2, p-ERK, cPLA$_2$ or VEGF antibodies overnight at 4°C and incubated for 1 h with secondary peroxidase-conjugated antibody. The membranes were developed with an enhanced chemiluminescence system (Amersham) and exposed to X-ray film (FUJI Photo Film, Tokyo, Japan). Quantification was carried out with a video densitometer (Scan Maker III, Microtek, USA).

**Cell viability**

Cell viability was measured using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method (23). After incubation with nicotine at different concentrations, cells were incubated with 2.5% MTT solution (5 mg/ml) for another 4 h at 37°C. Thereafter, 0.04 M...
HCl-isopropanol was added to the solution and mixed thoroughly. The color change was recorded using spectrophotometry with the microplate reader (MRX, Dynex Technologies, Sullyfield Circle Chantilly, USA) at 570 nm. The same test was repeated three times and the optical density was calculated for statistical analysis.

Statistical analysis

Results were expressed as the mean ± SD. Statistical analysis was performed with an analysis of variance (ANOVA) followed by the Tukey t-test. P values <0.05 were considered statistically significant.

Results

Nicotine promoted growth of human gastric xenograft tumor in nude mice

To examine the direct effect of nicotine in gastric tumorigenesis, AGS cells were inoculated into the gastric walls of the nude mice, which were given nicotine (50 or 200 μg/ml) in drinking water ad libitum. Tumors were found in the stomach of all the mice after 3 months (Figure 1). Malignancy was confirmed histologically by the H&E staining, and the tumor area (mm²) was found to be significantly larger (P < 0.01) in the nicotine-treated group (50 μg/ml, 25.34 ± 1.02 mm²; 200 μg/ml, 29.91 ± 2.23 mm²) than in the tumor control group (18.05 ± 3.27 mm²). Intraperitoneal injection of the selective COX-2 inhibitor, SC-236 (1 or 2 mg/kg), dose-dependently attenuated the gastric tumor area promoted by nicotine (Table I).

Cell proliferation in the gastric tissues

To delineate the underlying mechanism of nicotine in inducing gastric tumor growth, the increase in cell number in the tumor tissue was measured using proliferating cell nuclear antigen (PCNA) staining. Cell proliferation was increased significantly in tumor-bearing mice when compared with those without tumor (normal control group). Results also showed that treatment with nicotine caused a marked increase in the number of proliferative cells in gastric tissues when compared with the tumor control group (Table I). PCNA-positive staining of cells was denser in the low-dose (22.61 ± 1.73) and high-dose (28.56 ± 2.20) nicotine-treated groups than the tumor control group (16.02 ± 0.60). In the 1 and 2 mg/kg SC-236 groups, the inhibitory effect on cell proliferation was in accordance with reduced tumor area, and the higher dose of SC-236 exerted more potent inhibitory action on gastric tumor growth.

Microvessel density and its angiogenic growth factor VEGF in the gastric tissues

To further explore the underlying mechanism of nicotine in inducing gastric tumor growth, the formation of new blood vessels (angiogenesis) was measured. Results showed that only the high dose of nicotine significantly stimulated neo-vascularization, by ~30%, in the tumor tissues when compared with the tumor control group (Figure 2A), whereas the low-dose nicotine-treated group was not statistically

![Image](https://academic.oup.com/carcin/article-abstract/25/12/2487/2475918)

![Image](https://academic.oup.com/carcin/article-abstract/25/12/2487/2475918)

**Table I.** Effect of nicotine on tumor area (mm²) and cell proliferation using PCNA-staining in human gastric xenograft model

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight (g)</th>
<th>No. of mice with gastric tumors</th>
<th>Tumor area (mm²)</th>
<th>No. of PCNA-positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>25.15 ± 0.95</td>
<td>0/5</td>
<td>—</td>
<td>11.20 ± 2.19</td>
</tr>
<tr>
<td><strong>Tumor implantation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>22.22 ± 0.25</td>
<td>10/10</td>
<td>18.05 ± 3.27</td>
<td>16.02 ± 0.60a</td>
</tr>
<tr>
<td>SC-236 1 mg/kg</td>
<td>23.48 ± 1.04</td>
<td>10/10</td>
<td>17.54 ± 3.89</td>
<td>17.43 ± 0.84</td>
</tr>
<tr>
<td>SC-236 2 mg/kg</td>
<td>20.92 ± 0.92</td>
<td>10/10</td>
<td>14.47 ± 1.68</td>
<td>13.71 ± 1.10</td>
</tr>
<tr>
<td><strong>Low dose nicotine treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotine 50 μg/ml</td>
<td>22.26 ± 0.27</td>
<td>10/10</td>
<td>25.34 ± 1.02b</td>
<td>22.61 ± 1.73c</td>
</tr>
<tr>
<td>SC-236 (1 mg/kg)</td>
<td>23.96 ± 0.73</td>
<td>10/10</td>
<td>24.89 ± 2.48</td>
<td>19.15 ± 0.43d</td>
</tr>
<tr>
<td>SC-236 (2 mg/kg)</td>
<td>21.38 ± 0.42</td>
<td>10/10</td>
<td>20.83 ± 0.89d</td>
<td>16.28 ± 2.70f</td>
</tr>
<tr>
<td><strong>High dose nicotine treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotine 200 μg/ml</td>
<td>16.10 ± 1.83a,c</td>
<td>10/10</td>
<td>29.91 ± 2.23e</td>
<td>28.56 ± 2.20f</td>
</tr>
<tr>
<td>SC-236 (1 mg/kg)</td>
<td>20.06 ± 0.84d</td>
<td>10/10</td>
<td>20.54 ± 1.46e</td>
<td>24.80 ± 1.72d</td>
</tr>
<tr>
<td>SC-236 (2 mg/kg)</td>
<td>22.06 ± 0.24f</td>
<td>10/10</td>
<td>11.63 ± 1.11f</td>
<td>18.31 ± 0.92g</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD from 5–10 mice. aP < 0.05 versus normal group; bP < 0.05; cP < 0.01 versus control group; dP < 0.05; eP < 0.01; fP < 0.001 versus respective nicotine-treated control groups.
Nicotine stimulated neovascularization in gastric tumor tissues. Mice were given 50 (50 Nic) or 200 mg/ml (200 Nic) nicotine in drinking water with or without SC-236 i.p. injection (1 or 2 mg/kg, three times per week) for 3 months. (A) Microvessel density in gastric tumor tissues by immunostaining and (B) angiogenic factor VEGF expression by western blot. Data are presented as mean ± SD from 10 mice in each group. *P < 0.05 versus normal control group; †P < 0.05 versus respective nicotine-treated control groups.

![Image](https://example.com/image1.png)

**Fig. 2.** Nicotine stimulated neovascularization in gastric tumor tissues. Microvessel density (A) and VEGF expression (B) were assayed as described in the Methods section. Data are presented as mean ± SD from 10 mice in each group. *P < 0.05 versus normal control group; †P < 0.05 versus respective nicotine-treated control groups.

Roles of COX-2 in nicotine-induced gastric cancer growth in AGS cells

In order to better understand the role of nicotine in gastric tumorigenesis, gastric cancer cells (AGS) were used to evaluate the direct effect of nicotine on cell proliferation in vitro. AGS cells were cultured in the presence of nicotine (10-200 µg/ml) for 5 h and then the amount of DNA synthesis was measured. Results showed that nicotine significantly induced cell proliferation in a dose-dependent manner (Figure 5A), and exerted no cytotoxic effects on the cells (data not shown). At 50 µg/ml, nicotine increased cell proliferation by 10%, and the highest concentration of nicotine further stimulated gastric cancer cell growth by 40%.

Nicotine not only stimulated cell proliferation, but also strongly up-regulated COX-2 protein expression by ~2-fold when compared with the tumor control (Figure 5C). To confirm the role of COX-2 in cancer growth, a specific inhibitor of COX-2 activity (SC-236) was incubated with nicotine-treated cells. Nicotine-induced gastric cell proliferation was completely reversed by SC-236 (10 µg/ml) at all doses, and returned to basal levels (Figure 6A). Nicotine dose-dependently increased PGE2 release from AGS cells. SC-236 by itself did not affect the basal PGE2 level; however, it completely reversed the stimulatory action of nicotine (Figure 6B).

Involvement of ERK in nicotine-induced gastric cancer growth

Nicotine was found to stimulate gastric cancer growth in mice, and also in the AGS cell culture system. Our results showed that nicotine dose-dependently activated the phosphorylation of ERK, which was inhibited by its upstream inhibitors of MEK-1 (PD98059) and MEK-1/2 (U0126) (Figure 7B).
Both MEK inhibitors had a similar inhibitory effect on nicotine-induced cell proliferation of ~20% (Figure 7A).

To evaluate the correlation between ERK and COX-2 in nicotine-induced cell proliferation, protein expression of COX-2 was determined in AGS cells treated with MEK-1/2 inhibitors. Inhibition of nicotine-induced activation of COX-2 was observed after treatment with MEK inhibitors; however, U0126 attenuated nicotine-induced COX-2 up-regulation to a greater extent than PD98059 (Figure 7C). SC-236 had no detectable effects on ERK phosphorylation levels (data not shown). Thus, it is possible that nicotine-induced COX-2 up-regulation is mediated by ERK phosphorylation in gastric tumor growth.

Inhibitors of ERK or COX-2 attenuated nicotine-induced VEGF levels

The mechanism by which nicotine evokes a vasculogenic response is largely unknown. Activation of the ERK signaling pathway appears to play a crucial role in neovascularization. To determine whether the ERK/COX-2 pathway is related to angiogenesis, we measured the levels of VEGF in response to U0126 (MEK-1/2 inhibitor) or SC-236 (COX-2 inhibitor) in nicotine-treated gastric cancer cells. Nicotine significantly increased the levels of VEGF by ~70% over the tumor control group, which paralleled the increase in angiogenesis in the animal model. The increase in VEGF levels was attenuated by both the inhibitors of MEK (U0126) and COX-2 (SC-236) (Figure 8), suggesting that ERK and COX-2 are involved in nicotine-mediated VEGF expression.

Discussion

Here, we demonstrate for the first time that nicotine increases gastric tumor growth in mice with orthotopic gastric cancer cell implantation, a realistic model of gastric tumors. Our results showed that nicotine accelerated gastric cell proliferation (PCNA staining) and enhanced neovascularization (Table I, Figure 2). These mitogenic effects correlated well with the induction of COX-2, ERK phosphorylation and increased VEGF and PGE2 levels. All these observations are associated with the growth of gastric adenocarcinoma in human subjects.
Previous work showed that cigarette smoke and COX-2 are associated with gastric cell growth in vitro (24), but evidence for a direct effect of nicotine on gastric tumorigenesis was inadequate. Our findings revealed that nicotine promoted gastric tumor growth by increasing cell proliferation and microvessel density both in vivo and in vitro. Furthermore, these processes are COX-2- and VEGF-dependent. An expanding body of evidence reports that COX-2 is required for tumor growth and neovascularization (25) and its over-expression promotes gastric tumorigenesis (10–12). Inhibitors of COX-2 suppressed tumor growth in human cancer xenograft models through the induction of apoptosis and inhibition of cancer cell growth (26). The data suggest that COX-2 is a potential antitumor target for gastric cancer.

In our studies, nicotine-induced gastric cancer growth was associated with an increase in ERK phosphorylation and COX-2 expression in gastric cancer cells, and this effect could be attenuated by their respective inhibitors (Figures 6A and 7A). Other studies have shown that nicotine activates PKC and ERK phosphorylation in lung cancer cells and vascular cells (27,28). Nicotine not only increases the phosphorylation of ERK but also the downstream transcription factor cAMP response element binding protein in rat PC12 h cells (29). The nicotinic receptor antagonists, hexamethonium and decamethonium, suppress cancer growth and increase apoptosis (30). All these findings suggest strongly that nicotine acts as a promoter in the process of carcinogenesis, which is regulated by nicotinic receptor and MAPK activation. Notably, the present study revealed that nicotine treatment mediated the induction of COX-2 protein expression, which could be blocked by the MEK inhibitors (Figure 7C). Hence, the promotion effect of nicotine on gastric cancer growth is through the ERK/COX-2 pathway.

A selective inhibitor of MEK counteracts Helicobacter pylori-induced VEGF up-regulation, suggesting that VEGF and ERK are involved in gastric carcinogenesis (31). Nicotine stimulates endothelial cells to release prostanoids (32), which modulate the angiogenic factor VEGF (33). This finding supports the notion that COX-2-derived PGs and VEGF are required for vascularization. Consistent with
the above premise, we also demonstrated that blockers of MEK or COX-2 notably reduced the up-regulation of VEGF mediated by nicotine (Figure 8). However, it remains unproven that COX-2-derived PGs, which would act on tumor cells to release other growth factors and promote neovascularization in an autocrine fashion, is the ultimate explanation for gastric tumor growth. Hence, future studies are required to validate the full extent of COX-2-dependent processes in tumor cells or stroma cells in neovascularization.

In conclusion, the present study reveals significant effects of nicotine on COX-2 expression in gastric tumorigenesis. Furthermore, blockade of ERK can counteract nicotine-induced COX-2 expression, implicating the involvement of ERK/COX-2 in the tumorigenic action of nicotine in the stomach. In addition, inhibitors of the ERK and COX-2 pathway reduced VEGF levels, suggesting that VEGF is the downstream mediator for both ERK and COX-2 in promotion of neovascularization during tumor growth (Figure 9). Therefore, inhibitors that can block ERK/COX-2/VEGF-related pathways may be useful therapeutic agents for gastric cancer in cigarette smokers.
in confirming adenocarcinoma in gastric tissues and also Dr I.C. Bruce (Department of Pathology, Sun Yat-Sen University, China) for his assistance.

Grants from the University of Hong Kong and the Hong Kong Research Grants Council (HKU 7281/02 M). We would like to thank Professor Lin Han-Ling (Department of Pathology, Sun Yat-Sen University, China) for his assistance in confirming adenocarcinoma in gastric tissues and also Dr I.C. Bruce (Department of Physiology, the University of Hong Kong) for his comments on the manuscript.

References


Fig. 8. Effects of MEK inhibitor (U0126, 20 mM) and selective COX-2 inhibitor (SC-236, 10 μg/ml) on nicotine-mediated VEGF level. Cells were treated with SC-236 or U0126 for 60 min prior to nicotine treatment (200 μg/ml) for 5 h. Data are presented as mean ± SD of at least three independent experiments. *P < 0.01 versus control group; †P < 0.01 versus nicotine-treated group.

Nicotine

ERK —— U0126, PD98059

COX-2 —— SC-236

PGs

VEGF

Cell proliferation

Angiogenesis

Tumor Growth

Fig. 9. Proposed mechanism of nicotine-mediated processes of cell proliferation and angiogenesis during gastric tumor growth.

Acknowledgements

This study was supported by the Committee on Research and Conference Grants from the University of Hong Kong and the Hong Kong Research Grants Council (HKU 7281/02 M). We would like to thank Professor Lin Han-Ling (Department of Pathology, Sun Yat-Sen University, China) for his assistance in confirming adenocarcinoma in gastric tissues and also Dr I.C. Bruce (Department of Physiology, the University of Hong Kong) for his comments on the manuscript.

Received March 14, 2004; revised August 4, 2004; accepted August 10, 2004