Nicotine Promoted Colon Cancer Growth via Epidermal Growth Factor Receptor, c-Src, and 5-Lipoxygenase-Mediated Signal Pathway


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ABSTRACT

Nicotine [3-(1-methyl-2-pyrrolidinyl)-pyridine], a major alkaloid in tobacco, has been implicated as playing a role in carcinogenesis. Our previous study showed that passive cigarette smoking promoted inflammation-associated colonic adenoma formation in mice, and 5-lipoxygenase (5-LOX) plays an important role in this process. In the present study, we aimed to investigate whether nicotine could stimulate colon cancer cell proliferation and tumor growth in nude mice xenograft model and the possible mechanisms involved. Results showed that nicotine stimulated SW1116 colon cancer cell proliferation in a dose-dependent manner. Epidermal growth factor receptor (EGFR) and c-Src phosphorylation levels together with protein expression of 5-LOX were also significantly enhanced in this proliferation process. Inhibitors of EGFR and c-Src alleviated the actions of nicotine on cell proliferation and 5-LOX protein expression. Combination of both agents produced additive effect. In contrast, 5-LOX inhibitor had no direct effect on the phosphorylation levels of EGFR and c-Src and yet inhibited cell proliferation. In the colon cancer xenograft model, nicotine also significantly enhanced tumor growth. This acceleration of tumor growth corresponded well with increased vascularization and its proangiogenic factors. Inhibitors of EGFR, c-Src, and 5-LOX all significantly impeded the tumor growth induced by nicotine. Together, nicotine can promote colonic tumorigenesis both in vitro and in vivo. Activation of the phosphorylated form of EGFR and c-Src followed by an increased 5-LOX expression are the prime pathogenic mechanisms in the tumorigenic process in the colon.

Cigarette smoke is a major risk factor in cancer development. It causes most cancers of the lung, oropharynx, larynx, and esophagus, and approximately one-third of all cancers of the pancreas, kidney, urinary bladder, and uterine cervix. More recent evidence also implicates the relationship between smoking and cancers of the stomach, liver, and colon (Michael et al., 2002). Cigarette smoke is a complex mixture containing thousands of different compounds, of which 100 are known to be carcinogens, cocarcinogens, mutagens, and/or tumor promoters (Hoffmann et al., 2001). Cigarette smoke, which is a major alkaloid in tobacco, has been implicated as playing a role in carcinogenesis. It was implied that nicotine might be genotoxic based on the fact that large doses of nicotine could significantly increase mutation frequency and sister chromatid exchange frequency in cellular experiments (Riebe and Westphal, 1983; Trivedi et al., 1999). Li et al. reported that nicotine could form adducts with mouse liver DNA, lung DNA, histone H1/H3, Hb, and albumin in mice and demonstrated a positive relationship between dose and adduction (Li et al., 1996; Wu et al., 1997), whereas DNA adducts have been widely accepted as biomarkers for the dosimetry of chemical carcinogens. Furthermore, Pratesi et al. and others reported that nicotine stimulated the growth of human small cell lung cancer cells (Cattaneo et al., 1993; Codignola et al., 1994; Pratesi et al., 1996). It is also indicated that chronic exposure to nicotine can lead to sustained activation of growth-promoting pathways and may facilitate the development of cancer and potentially reduce the efficacy of anti-
cancer agents by activating survival pathways (Heusch and Maneckjee, 1998). However, the carcinogenic action of nicotine on the colon has not been defined.

Colorectal cancer is the fourth most common malignancy affecting the Western population and the second leading cause of deaths due to cancer. The genetic events leading to malignant disease have been well documented, allowing stepwise models of colonic tumorigenesis to be elucidated. In addition to these genetic anomalies, activation of the nonreceptor tyrosine kinase c-Src has been a consistent early finding in colorectal cancer (Bolen et al., 1987; Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996). A recent report of activating c-Src mutation in a small subset (12%) of highly advanced colon tumors has verified an important role for c-Src in the progression of colorectal cancer (Irby et al., 1999). It is also noted that overexpression of epidermal growth factor receptor (EGFR), the most commonly observed cancer-associated misregulation in EGFR signaling, correlates with poor prognosis in a number of cancers (Dassonville et al., 1993; Tewari et al., 2000; Umekita et al., 2000). In the gastrointestinal tract, expression of EGFR and its ligands is often higher in tumors than in surrounding normal tissues (Messa et al., 1998). Furthermore, the level of EGFR expression generally correlates with colon cancer progression and metastatic potential (Hayashi et al., 1994; Radinsky et al., 1995). In tumor cells, EGFR polarity may be lost, providing an additional avenue for altered EGFR action influencing abnormal cell growth (Tong et al., 1998). Interestingly, nicotine was reported to affect this growth factor receptor (Wang et al., 1996; Mathur et al., 2000; Nakayama et al., 2002). Similar stimulating action on colon cancer cells has not been identified. Our previous study showed that passive cigarette smoking promoted inflammation-associated colonic adenoma formation in mice (Liu et al., 2003) and 5-lipoxygenase (5-LOX) plays an important role in this process (Ye et al., 2003). The association between EGFR and c-Src with 5-LOX has often been suggested in the pancreatic adenocarcinoma development (Schuller et al., 2002). Therefore, in the present study, we aimed to investigate whether nicotine could stimulate colon cancer cell proliferation and tumor growth in nude mice, and the possible involvement of EGFR, c-Src together with 5-LOX in this pathogenic cascade.

Materials and Methods

Cell Culture and Chemicals. SW1116 cells, a cell line derived from human colon adenocarcinoma, were obtained from American Type Culture Collection (Manassas, VA) and were cultured in Leibovitz's L-15 medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. AG1478 and PP2 were purchased from Calbiochem (San Diego, CA), and MK886 was kindly provided by Merck Frosst (Montreal, Canada). 5-LOX monoclonal antibody was purchased from BD Transduction Laboratories (Lexington, KY), and EGFR and c-Src antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Estimation of Cell Viability. Cell viability was estimated using MTT assay. Simply, SW1116 cells were seeded into a 96-well plate and incubated for 24 h for attachment. They were then incubated with different drugs for different times at 37°C. At the end of incubation, the medium was aspirated. The remaining cells were further incubated with 0.25 mg/ml of MTT for 3 h. MTT was extracted with isopropanol in 0.04 M hydrochloric acid, and the color change in the extract was measured at 595 nm.

Measurement of Cell Proliferation. Cell proliferation was assayed as DNA synthesis. To evaluate DNA synthesis in cells, the incorporation of [3H]thymidine into DNA was determined. Briefly, cells were seeded into a 24-well plate and cultured for 24 h for attachment. They were then washed twice with 0.01 M phosphate-buffered saline, followed by incubation with 1 ml/well of the medium containing various substances for different times. In the next step, 0.5 μCi of [3H]thymidine was added to each well, and the cells were further incubated for 5 h. Incorporation of [3H]thymidine into cells was measured with a liquid scintillation counter (LS-6500; Beckman Coulter, Inc., Fullerton, CA).

Tumorigenicity in Nude Mice. Single cell suspensions of SW1116 cell lines were typsized and collected. The cell viability was >95% as determined by trypsin blue staining. Cells (3 × 10⁵) in a 0.2-ml volume of phosphate-buffered saline were inoculated subcutaneously into the right flank of 4-week-old female BALB/c nu/nu mice (Laboratory Animal Unit, The University of Hong Kong). The mice were maintained under sterile conditions. Once palpable tumors were established, tumor volume was calculated as follows: V = (4/3)π R1²R2, where R1 is radius 1 and R2 is radius 2 and R1 < R2. According to the Animal nude mice Ethics, the mice were sacrificed in 3 weeks. At the end of the experiments, the tumors were excised and kept in 4% formalin or liquid nitrogen for immunohistochemistry or Western blot analysis.

Western Blot. The cells and the tumors were harvested at 4°C with radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 0.5% a-cholate, 0.1% SDS, 2 mM EDTA, 1% Triton X-100, and 10% glycerol) containing 1.0 mM phenylmethylsulfonyl fluoride and 1 μg/ml aprotinin. After sonicated or homogenized on ice, the samples were then centrifuged at 17,968g for 20 min at 4°C and the supernatant containing 70 μg of the protein was denatured and separated by electrophoresis on a sodium dodecyl-polyacrylamide gel. The protein was then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) that was probed with respective primary antibody. Membranes were developed by the ECL chemiluminescence and exposed on X-ray film. Quantification of the bands on the film was carried out by video densitometry (Gel Doc 1000; Bio-Rad).

Immunoprecipitation. Cells and tumor tissues were lysed in a lysis buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate, 5 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μM antipain, and 5 μg/ml aprotinin) and clarified by centrifugation. The protein concentration of the lystate was determined using a bicinechonic acid protein assay kit (Pierce Chemical, Rockford, IL). Equal amounts of protein were incubated with specific antibody immobilized onto protein A-Sepharose for 2 h at 4°C with gentle rotation. Beads were washed extensively with lysis buffer and immune complexes were eluted in 2× Laemmli buffer, boiled, and microcentrifuged. Proteins were resolved on SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and incubated with specific primary antibodies. After washing and incubating with secondary antibodies, immunoreactive proteins were visualized by the ECL detection system (Amersham Biosciences Inc., Piscataway, NJ). Where indicated, the membranes were stripped and reprobed with another antibody.

Assessment of Angiogenesis in Tumors. The microvessels in the colonic mucosa were identified by immunohistochemical staining with von Willebrand factor antibody (DAKO, Carpinteria, CA). The colonic tissues were fixed and then followed by deparaffinization and hydration. After incubation with 0.3% H₂O₂/methanol, the sections were subjected to trypsin digestion for 30 min and then blocked by the addition of normal serum in 0.05 M Tris-HCl buffer saline (Tris-buffered saline; pH 7.8) for an hour. They were then incubated overnight with von Willebrand factor (1:200) at 4°C. The endothelial cells in the blood vessels were labeled by the addition of peroxidase-
conjugated streptavidin followed by 3,3′-diaminobenzidine. The number of blood vessels in the colonic tissue was counted using a light microscope and expressed as the number of blood vessel per square millimeter.

Statistical Analysis. Student’s t test was used to compare data between two groups. One-way analysis of variance and Bonferroni correction were used to compare data between three or more groups. Values are expressed as mean ± S.E.M. P < 0.05 was considered statistically significant.

Results

Effect of Nicotine on Colon Cancer Cell Proliferation. Figure 1 shows that nicotine, a major alkaloid in tobacco products, stimulated SW1116 colon cancer cell proliferation in a dose-dependent manner with a maximum effect occurring at the dose of 1000 nM after incubation for 5 h. At this dose, the increased level of cell proliferation promoted by nicotine was 40% higher than that of the control group.

Effect of Nicotine on EGFR, c-Src, and 5-LOX Levels in Colon Cancer Cells. EGFR and c-Src protein expressions in SW1116 colon cancer cells were not changed after incubation with 1000 nM nicotine for 5 h, but their phosphorylation levels were significantly enhanced. This was accompanied by the increased protein expression of 5-LOX (Fig. 2).

Effect of EGFR and c-Src Inhibitors on Colon Cancer Cell Proliferation Induced by Nicotine. To address the role of EGFR and c-Src in the cell proliferation induced by nicotine, we pretreated colon cancer cells with EGFR tyrosine kinase inhibitor AG1478 and/or c-Src inhibitor PP2 1 h before the addition of nicotine. Figure 3 shows that AG1478 at the dose of 200 nM significantly inhibited the cell proliferation induced by 1000 nM nicotine; further reduction was not found at the higher dose of 1 μM. Similar result was found with PP2 at the doses of 50 and 250 nM. It also partially reduced nicotine-induced colon cancer cell proliferation. Interestingly, copretreatment of colon cancer cells with AG1478 (200 nM) and PP2 (50 nM) inhibited cell proliferation to a much lower level compared with those treated with either AG1478 (200 nM) or PP2 (50 nM) alone. MTT assay showed that the doses of AG1478 and PP2 used in the present study had no cytotoxic effect on colon cancer cells (data not shown).

Effect of EGFR and c-Src Inhibitors on Colon Cancer Cell Protein Expression and Phosphorylation Levels Induced by Nicotine. Furthermore, AG1478 and PP2, respectively, inhibited the stimulating effect of nicotine (1000 nM) on 5-LOX protein expression. Their combined effect was more significant. In addition, we also found that AG1478 decreased the stimulatory effect of nicotine on c-Src phosphorylation level, whereas PP2 produced the similar effect on the phosphorylation level of EGFR in cancer cells (Fig. 4).

Effect of 5-LOX Inhibitor on Colon Cancer Cell Proliferation and Phosphorylation Levels of EGFR and c-Src Induced by Nicotine. Pretreatment of SW1116 cells with MK886, a specific inhibitor of 5-LOX, for 1 h partially decreased colon cancer cell proliferation induced by nicotine at the dose of 1000 nM for 5-h incubation (Fig. 5). However, the phosphorylation levels of EGFR and c-Src were not modified by the treatment of MK886 (data not shown).

Effect of Nicotine on Tumor Growth in Xenograft Model. We had demonstrated that nicotine was able to directly stimulate cell proliferation in colon cancer cell line. To determine whether nicotine could promote tumor growth in animals, we used the in vivo colon cancer xenograft model. The colon cancer cells were treated with 10, 100, and 1000 nM nicotine for 5 h, and then inoculated into the skin of the nude mice. Ten days after implantation of colon cancer cells, all mice had similar tumor size. At day 21, however, tumor growth in the nicotine-treated groups markedly exceeded that in the control group and required killing of the mice. The present study also demonstrated a dose-dependent effect of nicotine on tumor growth. On day 21, the tumor volume was 0.95 cm³ in the control group, 1.82 cm³ in the “10 nM nicotine” group, 2.71 cm³ in the “100 nM nicotine” group, and 3.82 cm³ in the “1000 nM nicotine” group (Fig. 6). Furthermore, the phosphorylation levels of EGFR, c-Src, and protein expression of 5-LOX were also significantly enhanced by 6-, 4.5-, or 5-fold, respectively, in the tumor tissues treated with 1000 nM nicotine compared with the control group.

In addition, this acceleration of tumor growth in the nicotine-treated groups corresponded well with an increased vascularization. At the time of tumor explantation (on day 21), we observed a significantly higher capillary density in the tumors when exposed to 1000 nM nicotine (Fig. 7). Moreover, the tissue levels of VEGF, MMP-2, and MMP-9 at the tumors were significantly higher in the nicotine-treated groups compared with the control group (Fig. 8).

Effect of Inhibitors of EGFR, c-Src, and 5-LOX on Tumor Growth Induced by Nicotine. To confirm the contributory role of EGFR, c-Src, and 5-LOX in the promotion of tumor growth, colon cancer cells were pretreated with AG1478, PP2, or MK886 for 1 h, respectively, or in combination before incubation with 1000 nM nicotine for 5 h and then inoculated into the skin of nude mice. Table 1 shows that both AG1478 and PP2, respectively, inhibited the stimulatory action of nicotine on tumor growth, and the effect was more significant when the two inhibitors combined. MK886, the inhibitor of 5-LOX, also reduced the tumor size of nicotine-treated group. The tumor size was, however, greater than that cotreated with AG1478 and PP2, but smaller than those treated with AG1478 or PP2 alone.

Discussion

Nicotine is generally accepted to be an active alkaloid in tobacco, typically comprising of 1 to 2% weight of tobacco. It
is a dominant factor for tobacco addiction. In the present study, the stimulatory effect on human colon cancer cell proliferation was observed after nicotine treatment as low as 10 nM. The nicotine effect was dose-dependent, with maximal stimulation seen with 1000 nM nicotine after 5-h exposure. These concentrations of nicotine were similar to the amount of nicotine intake in cigarette smokers (Benowitz, 1988; Armstrong et al., 1996). However, it is still unknown whether the action is mediated through direct receptor acti-
In the present study, we found that in the process of cell proliferation induced by nicotine, the protein expressions of EGFR and c-Src were not changed. But their phosphorylation levels were significantly enhanced. Blockade of this phosphorylation process respectively inhibited cell proliferation by AG1478 and/or PP2. These findings demonstrated that the nicotine was acting via EGFR and c-Src activation in human SW1116 colon cancer cells. EGFR and c-Src are the prototypes that individually represent the receptor and non-receptor tyrosine kinases that control different cellular activities. Accumulated evidence reveals the association between EGF-activated EGFR and c-Src that leads to activation of both kinases. Importantly, their mutual interaction is required for many EGFR-mediated cellular functions, including proliferation, migration, survival, and EGFR endocytosis (Leu and Maa, 2003). In the current study, we also found that the two kinases maintained a bidirectional interaction: inhibition of either EGFR or c-Src decreased the reciprocal phosphorylation level of c-Src or EGFR in the colon cancer cells. In addition, cotreatment of the cells with EGFR and c-Src inhibitors reduced cell proliferation to a much lower level compared with those treated with the respective inhibitor. This confirms the collaboration role of EGFR and c-Src in the induction of cell proliferation by nicotine. Similar result was also found in the animal model. The tumor size of the “AG1478 and PP2-cotreated group” was significantly smaller than those treated with the individual inhibitor alone. In addition, our preliminary study showed that there was alpha (7) nicotinic acetylcholine receptor mRNA expression in SW1116 colon cancer cells. This was significantly enhanced by nicotine by 3-fold. Nicotinic receptor antagonist mecamylamine partially decreased SW1116 cell proliferation induced by nicotine. However, the relationship between nicotine acetylcholine receptor stimulation and expression of EGFR and c-Src remains to be studied.

Our results also showed that both inhibitors of EGFR and c-Src reduced 5-LOX protein expression in the process of cell proliferation enhanced by nicotine, whereas 5-LOX inhibitor had no direct modification effect on the phosphorylation levels of EGFR and c-Src. These results suggest that 5-LOX may be the down-stream mediator of the EGFR/c-Src signal pathway in colon cancer cell proliferation. This hypothesis is supported by the experimental evidences that a short, praline-rich region spanning residues 566 to 577 in human 5-LOX is a binding site for the c-Src homology 3 domain of growth factor receptor-bound protein 2, an “adaptor” protein for tyrosine kinase-mediated cell signaling (Lepley and Fitzpatrick, 1994). In the animal model, the tumor size of 5-LOX inhibitor-treated group was larger than that of AG1478 and PP2-cotreated group, but smaller than those treated alone. This would further confirm the coregulatory role of EGFR/c-Src in the tumorigenesis of colon cancer, and it was mainly but not wholly through the activation of 5-LOX expression in colon cancer cells.
Our previous study indicated that passive cigarette smoking promoted inflammation-associated adenoma formation in the colon through activation of angiogenesis and VEGF/MMP-2 pathway (Ye et al., 2003). Experimental evidence shows that VEGF and MMPs play a significant role in the angiogenesis and metastasis processes during cancer development (Deryugina et al., 2002; Sounni et al., 2002; Somlyo et al., 2003). In this study, not only the levels of EGFR and c-Src were determined and increased by nicotine but also these proangiogenic factors were measured during tumor growth. It is because short-term exposure to nicotine might lead to a more angiogenic phenotype in the SW1116 cells that would account for more rapid tumor growth in the later stages of the experiment. Consequently, we found that the acceleration of tumor growth induced by nicotine corresponded well with an increased vascularization at the tumor site. Moreover, the tumor levels of VEGF, MMP-2, and MMP-9 were significantly higher in this group compared with the control group. These results further confirm that in addition to an increase in cell proliferation as reported in the early part of this study, the proangiogenic effect of nicotine is another contributory factor in the promotion of tumor growth in the colon. It would be interesting to see the impact of continuous nicotine exposure on tumor growth after the implantation of colon cancer cells.

In summary, the findings reported here identify a novel nicotine-stimulated survival signal pathway that involves phosphorylation of EGFR and c-Src followed by an increased 5-LOX expression in human SW1116 cells. These intriguing findings strongly suggest a novel strategy for the treatment of human colon cancer by blocking the EGFR, c-Src, and 5-LOX cascade.

### References


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