Reduction of EGF is associated with the delay of ulcer healing by cigarette smoking

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Ma, L., W. P. Wang, J. Y. C. Chow, S. T. Yuen, and C. H. Cho. Reduction of EGF is associated with the delay of ulcer healing by cigarette smoking. Am. J. Physiol. Gastrointest. Liver Physiol. 278: G10–G17, 2000.—Cigarette smoking is associated with peptic ulcer diseases. Smokers have lower levels of salivary epidermal growth factor (EGF) than nonsmokers. We investigated whether reduction of EGF is involved in the delay of gastric ulcer healing by cigarette smoking. Rats with acetic acid-induced ulcers were exposed to cigarette smoke (0, 2, or 4% vol/vol) 1 day after ulcer induction. EGF level was elevated 1 day after ulcer induction in salivary glands and serum, and 4 days after ulcer induction in the gastric mucosa. However, cigarette smoke depressed these beneficial effects and EGF mRNA expression in salivary glands and gastric mucosa. Cigarette smoke delayed gastric ulcer healing and reduced cell proliferation, angiogenesis, and mucus synthesis. Exogenous EGF (10 and 20 µg/kg iv) before smoke exposure reversed the adverse effects of cigarette smoke, whereas vascular endothelial growth factor level and nitric oxide synthase activity were unaffected. It is concluded that the detrimental effect of cigarette smoke on ulcer healing is a consequence of reduction of angiogenesis, cell proliferation, and mucus secretion through the depressive action on EGF biosynthesis and its mRNA expression in salivary glands and gastric mucosa.

epidermal growth factor; angiogenesis; proliferation; mucus; nitric oxide; vascular endothelial growth factor

Epidemiological and animal studies have shown that cigarette smoking is associated with the occurrence and recurrence of peptic ulcer diseases and the delay of ulcer healing, yet the mechanisms have not been fully elucidated. Ulcer healing is an active and complicated process that includes inflammation, tissue formation (granulation tissue formation, angiogenesis, and reepithelialization), and tissue remodeling. The critical event of ulcer healing is tissue formation, which requires the concerted interaction of various growth factors and cellular systems, such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and nitric oxide (NO) (15, 16, 25). EGF is mainly synthesized and secreted from the salivary glands under normal physiological conditions and is also produced by the "ulcer-associated cell lineage" after mucosal damage (32). It provides a variety of gastrointestinal protective effects and accelerates ulcer healing. EGF has been shown to promote not only epithelial restitution but also proliferation. When an ulcer develops, epithelial cells in the mucosa adjacent to the ulcer crater synthesize and secrete EGF, overexpress EGF receptor, and proliferate. Removal of salivary glands was found to delay ulcer healing, whereas supplementation of EGF subcutaneously completely reversed the delay in ulcer healing in sialoadenectomized rats (13). In addition, the level of salivary EGF was significantly lower in smokers than in nonsmokers (13). This could partially explain why cigarette smokers have a low ulcer healing rate in the ulcerated stomach.

During ulcer healing, angiogenesis is an important process that facilitates the delivery of nutrients, oxygen, and required growth factors to the proliferating tissue during the healing process. EGF has been demonstrated to have angiogenic effects both in vitro and in vivo (7, 23). Our previous finding (19) showed that cigarette smoke exposure delayed ulcer healing by decreasing angiogenesis, which was associated with a reduction of constitutive NO synthase (dNOS) activity. Furthermore, the interaction between EGF and NO during ulcer healing has been demonstrated. The healing effect of EGF on stress ulcers was completely reversed by Nω-nitro-L-arginine methyl ester (L-NAME), indicating that NO interacts with EGF in the recovery of mucosal injury (3). Also, EGF was found to increase the secretion and mRNA expression of VEGF, another potent angiogenic factor, in cell lines (27, 34). Thus it is plausible that cigarette smoking could not only adversely affect mucosal EGF expression but also alter the synthesis of NO and VEGF during the ulcer healing process in the stomach.

Gastric mucus is also important for tissue formation during ulcer healing. It provides a neutral pH environment that promotes epithelial restitution (30) and enhances the binding of EGF and other growth factors to their receptors (24). This could result in increased cell proliferation, leading to granulation tissue formation and reepithelialization (24). EGF by itself has been demonstrated to stimulate gastric mucus synthesis (11, 35). In contrast, sialoadenectomized rats had reduced levels of gastric mucus (22). Cigarette smoking could impair EGF release from salivary glands (16) and mucus synthesis in gastric mucosa (8). All these could be responsible for the adverse action of cigarette smoking on gastric ulcer healing. We therefore studied the
effects of cigarette smoking on mucosal EGF, NO, and VEGF expression in the gastric mucosa after ulcer induction. The effects of EGF on mucosal cell proliferation, angiogenesis, and mucus synthesis in connection with ulcer healing under the influence of cigarette smoking were also investigated.

MATERIALS AND METHODS

Animals and induction of gastric kissing ulcers. The present study was approved by the Committee on the Use of Live Animals for Teaching and Research at the University of Hong Kong. Male Sprague-Dawley rats (180–200 g) were reared on a standard laboratory diet (Ralston Purina, Chicago, IL) and given tap water. They were kept in a room where temperature (22 ± 1°C), humidity (65–70%), and day-night cycle (12:12-h light-dark) were maintained constant. Rats were deprived of food but had free access to tap water 24 h before ulcer induction. Gastric kissing ulcers were produced by luminal application of an acetic acid solution as described by Tsukimi and Okabe (28), with some modifications (19). Thereafter, animals were returned to a standard diet and given tap water ad libitum.

Cigarette smoke exposure. Unfiltered cigarettes (Camel, 1.2 mg of nicotine and 18 mg of tar per cigarette; R. J. Reynolds, Winston-Salem, NC) were used throughout the study. Intact or ulcerated rats (24 h after ulcer induction) were exposed to cigarette smoke (0, 2, or 4% vol/vol) in a chamber for a 1-h period once daily for various numbers of days. Procedures for cigarette smoke exposure and equipment used were described previously (4). The smoke exposure procedure used in this study does not affect the normal physiological functions of rats such as acid-base balance and O2-CO2 in blood, heart function, angiogenesis, and mucus synthesis in connection with ulcer healing under the influence of cigarette smoking were also investigated.

Measurement of NOS activity in the gastric mucosa. The tissue samples (100–150 mg) were homogenized for 20 s under ice-cold conditions in a buffer solution (10 mM HEPES, 320 mM sucrose, 0.1 mM EDTA, 1 mM dithiothreitol, 10 mg/ml soybean trypsin inhibitor, 2 mg/ml leupeptin, 2 mg/ml aprotinin, and 1 mg/ml phenylmethylsulfonyl fluoride), followed by centrifugation at 20,000 g for 20 min at 4°C. VEGF level in the supernatant was measured by an ELISA kit (Chemicon International, Temecula, CA). The coefficients of variation within and between assays were 8.0 and 11.9%, respectively. Protein concentration of the supernatant was determined. The final values were represented as picograms per milligram of protein for EGF in the gastric mucosa, nanograms per milligram of protein in the salivary gland, or nanograms per milliliter in the serum.

Assessment of mucosal cell proliferation. Mucosal cell proliferation was assessed by immunohistostaining as described by Tarnawski et al. (26). After incubation with HCl (2 M) solution for 1 h and neutralization with sodium borate (0.1 M, pH 8.6), the sections were trypsinized for 30 min at room temperature and subsequently blocked by normal serum for 1 h. They were then incubated with mouse anti-bromodeoxyuridine (BrdU) antibody (1:500) overnight at 4°C. After washing with PBS, a second antibody was applied for 1 h at room temperature. The sections were further washed and incubated with avidin-biotin complex (Vector Stain ABC kit; Vector Laboratories, Burlingame, CA) for another hour. Thereafter, the sections were counterstained with Mayer’s hematoxylin for 30 s, cleared by graded ethanol and xylene solutions, and then mounted. The BrdU-positive cells were detectable as a dark brown color in the nucleus after dianobenzidine reaction. They were counted under an image analyzer (Q500IW, Leica) by a person who was blind to the type of treatment. The final values were represented as the percentage of BrdU-positive cells.

Determination of angiogenesis in the granulation tissue at ulcer margin. Angiogenesis was assessed by counting the number of neomicrovessels using an immunohistocchemical method for von Willebrand factor expression (31) on 5-μm-thick sections. The neomicrovessels could be visualized after dianobenzidine reaction. They were counted under an image analyzer (Leica) at a ×200 magnification by a person...
who was blind to the type of treatment. Each slide was counted twice. Any brown-stained endothelial cell or endothelial cell cluster that was clearly separated from adjacent microvessels was considered a single, countable microvessel. The whole procedure was confirmed by a pathologist. The final values were represented as the number of microvessels per square millimeter in granulation tissues at the ulcer margin.

Assessment of mucosal mucus content. Sections were stained with periodic acid-Schiff and counterstained with Mayer’s hematoxylin. The amount of mucus within the mucosa was assessed by measuring the relative thickness of the mucus-secreting layer (2) using an image analyzer (Leica) at a 400× magnification, by a person who was unaware of the treatments. This method was based on the determination of the length of the gastric pit and isthmus (yi) over the total mucosal thickness (y), finally expressed as a ratio of x to y. In normal stomach sections, ten fields of measurements were taken and the results were averaged, whereas in ulcerated sections, two corresponding fields starting from the ulcer margins were measured and averaged.

Total RNA isolation. The salivary glands and gastric mucosa at the ulcer margin were lysed by homogenizing in 2 ml of TRIzol Reagent (GIBCO BRL, Gaithersburg, MD). Five minutes later, 0.4 ml of chloroform was added to each sample. The sample tubes were capped and shaken vigorously for 15 s, followed by centrifugation at 12,000 g for 15 min at 4°C. The RNA-containing aqueous phase was transferred to a fresh tube and mixed with 1 ml of isopropyl alcohol. The sample was then centrifuged at 12,000 g for 10 min at 4°C. The RNA pellet was washed with 75% ethanol, dried, and redissolved in 200 µl of diethyl pyrocarbonate water.

Determination of EGF mRNA expression by RT-PCR. Four micrograms of total RNA was used to generate the first strand of cDNA by RT (GIBCO BRL) according to the manufacturers’ instructions. PCR was then performed for EGF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from the same cDNA samples, using the following primer pairs (GIBCO BRL): for EGF, sense primer 5'-ATCTACCGCGGCGAGTCT-TCCAC-3' and antisense primer 5'-GCCAGCTTCCAC-CAACGTAAG-3'; corresponding to nt 3331–3352 and 3445–3466, respectively, of rat precursor protein (ppEGF) cDNA (21); and for GAPDH, sense primer 5'-ATTCTACCGCGGCGAGTCT-TCCAC-3' and antisense primer 5'-AGGGGCGGAGATGACGGACC-3', corresponding to nt 173–197 and 377–396, respectively, of rat GAPDH cDNA. PCR amplification was initiated by 5 min of denaturation at 94°C followed by 30 cycles consisting of the following phases: 1 min at 94°C (denaturation), 2 min at 61°C (annealing), and 3 min at 72°C (primer extension) using a PCR Core Kit (Boehringer Mannheim, Mannheim, Germany). After the final cycle of amplification, the samples were incubated for 7 min at 72°C. The PCR products (a 136-bp ppEGF fragment and a 224-bp GAPDH fragment) were then visualized by ultraviolet illumination after electrophoresis through 1% agarose gels containing 0.5 µg/ml ethidium bromide. The gel photographs were submitted to a multianalyst (Bio-Rad, Hercules, CA), and semiquantitative analysis of EGF mRNA was performed by comparing the intensity of the PCR product of EGF to that of GAPDH.

Statistical analysis. Results were expressed as means ± SE. Statistical analysis was performed with ANOVA and the unpaired Student’s t-test. P values <0.05 were considered statistically significant.

RESULTS

Effect of cigarette smoke exposure on EGF levels in saliva, serum, and gastric mucosa. As shown in Fig. 1A, the EGF level in serum was low in the intact animals. However, ulcer induction dramatically elevated and maintained the serum EGF at high levels during the experimental period. Nevertheless, the mucosal EGF level was not increased 1 day after ulcer induction. At 4 days, the amount of mucosal EGF became significantly higher than that in intact animals. Cigarette smoke exposure significantly decreased the EGF levels in both serum and gastric mucosa 4 days after ulcer induction (Fig. 1, A and B). Ulcer

![Fig. 1. Effect of cigarette smoke exposure on epidermal growth factor (EGF) levels in serum (A), gastric mucosa (B), and salivary glands (C). Rats were exposed to cigarette smoke (0, 2, or 4%) starting 1 day after ulcer induction for a 1-h period once daily for 3 or 6 days. Values are means ± SE of 8–10 rats. *P < 0.05, **P < 0.01 vs. corresponding 0% group before ulcer induction; †P < 0.05 vs. corresponding 0% group.](http://ajpgi.physiology.org/Downloadedfrom)
induction alone also markedly increased EGF levels in the salivary gland throughout the 7-day experimental period, which was not significantly affected by cigarette smoke exposure (Fig. 1C).

Effect of cigarette smoke exposure on EGF mRNA expression in salivary glands and gastric mucosa at ulcer margin. EGF content in the salivary gland was not affected by cigarette smoke exposure 4 days after ulcer induction, although serum EGF level, an indicator of EGF secretion from salivary glands, was significantly decreased by cigarette smoking (Fig. 1, B and C). Comparison of EGF mRNA levels in the smoking group (Fig. 2A, lanes 1 and 2) to those of the nonsmoking group (lanes 3 and 4) using RT-PCR revealed that cigarette smoke exposure significantly decreased EGF mRNA expression in the salivary glands. The mucosal EGF mRNA transcripts in the ulcer margin were also reduced in the smoking group (lanes 1–3) compared with those of the nonsmoking group (lanes 4–6).

Effects of cigarette smoke exposure on gastric ulcer healing, mucosal cell proliferation, angiogenesis, and mucus synthesis and their modifications by EGF. Mucosal cell proliferation was assessed by determining the ratio of BrdU-positive cells in the gastric mucosa. It was found that the number of proliferating cells was maximal at the ulcer margin (22.75 ± 1.68% on day 4 and 17.22 ± 1.00% on day 7) and gradually decreased toward the normal mucosa (6.42 ± 0.40% on day 4 and 5.73 ± 0.34% on day 7) in the control group. Cigarette smoke exposure for a 1-h period once daily for 3 or 6 days dramatically depressed gastric mucosal cell proliferation in a dose-dependent manner (Fig. 3A). The present study also confirmed our previous finding (19) that cigarette smoke delayed gastric ulcer healing (Fig. 4) and suppressed angiogenesis in the granulation tissue (Fig. 5) at the ulcer margin. In contrast, supplementation of EGF (10 and 20 µg/kg iv) completely reversed the inhibition of cigarette smoke on ulcer healing (Fig. 4), angiogenesis (Fig. 5), and mucosal cell proliferation (Fig. 3B). The same treatment, however, did not significantly enhance the healing rate in nonsmoking animals. The ulcer sizes in rats receiving EGF (20 µg/kg) without cigarette smoke exposure were 63.5 ± 3.6 and 30.0 ± 2.0 mm² on day 4 and day 7, respectively, which were comparable to the control without EGF treatment. In ulcerated rats, the thickness of the granular mucus-secreting layer 4 and 7 days after ulcer induction was maximal at the ulcer margin and decreasing toward the normal area, until it was similar to that of normal mucosa (Fig. 6, A and B). Cigarette smoke exposure substantially reduced the thickness of the mucus-secreting layer along the mucosal section (Fig. 6). Similarly, this inhibiting effect could also be reversed by exogenous EGF (Fig. 6).

Effects of cigarette smoke exposure on gastric mucosal VEGF level and NO activity and their modifications by EGF. VEGF and NO play an important role in angiogenesis. It was found that cigarette smoke decreased mucosal cNOS activity both 4 and 7 days after ulcer induction. However, exogenous EGF at the doses that completely reversed the inhibition of angiogenesis by cigarette smoke exposure did not restore cNOS activity (Fig. 7). On the other hand, neither cigarette smoke exposure nor exogenous EGF affected VEGF levels in the gastric mucosa (Fig. 8).

DISCUSSION

Ulcer healing is an active and complicated process that is composed of granulation tissue formation, angiogenesis, and reepithelialization. It requires concerted interaction of a variety of tissues and cellular systems,
including EGF, NO, and VEGF, which are involved in cell proliferation and angiogenesis during ulcer healing (15, 16, 25). Both clinical and animal studies have demonstrated that cigarette smoking delays gastric ulcer healing (10, 17). However, the underlying mechanisms are far from clear. In our animal smoking model, cigarette smoke exposure for a 1-h period once daily for 3 or 6 days significantly delayed gastric ulcer healing (Fig. 4). Although suppression of cNOS and angiogenesis in the granulation tissue has accounted for the delay of gastric ulcer healing, other mechanisms have also been suggested to be responsible for this action (19).

It has been shown that both salivary and circulating EGF contribute significantly to ulcer healing. EGF is mainly synthesized in and secreted from the salivary glands. Its level in blood is low under normal physiological conditions. However, ulcer induction alone dramatically elevated salivary gland and serum EGF levels 1 day after ulcer induction (Fig. 1A), indicating that mucosal damage could lead to EGF synthesis and secretion in salivary glands. This is in accord with the observation that EGF content in the submandibular glands was also significantly elevated after exposure to stress, a stimulus known to cause ulceration in rats (6). Furthermore, a significant increase of EGF level in the gastric mucosa was observed 4 days after ulcer induction (Fig. 1B). This delayed response is possible because of the local synthesis of EGF by a novel ulcer-associated cell lineage (32) in the gastric mucosa. Cigarette smoke exposure for a 1-h period once daily for 3 and 6 days not only significantly delayed gastric ulcer healing (Fig. 4).
but also significantly blocked the elevation of EGF in both serum and the gastric mucosa 4 days after ulcer induction (Fig. 1, A and B). Removal of the salivary glands was found to delay ulcer healing, whereas supplementation of EGF completely reversed the delay in ulcer healing (16). Clinical study also demonstrated that the healing rate in patients receiving 6 µg of EGF intravenously twice a week was significantly higher than that in patients receiving other antiulcer drugs (9). Because the salivary gland is the main source of serum EGF (20, 33), reduction of serum EGF levels after cigarette smoke exposure (Fig. 1A) could result from the suppression of EGF secretion from salivary glands. Furthermore, EGF mRNA levels in the salivary gland 4 days after ulcer induction was markedly depressed by cigarette smoke exposure (Fig. 2), indicating that EGF biosynthesis in the salivary gland was also decreased. Therefore, the unchanged EGF content in the salivary gland (Fig. 1C) could be explained by the net result from the depressed EGF synthesis in the salivary gland and the reduced EGF secretion from the salivary gland. EGF mRNA expression in the gastric mucosa was similarly reduced. All these findings suggested that inhibition of EGF synthesis might be one of the mechanisms by which cigarette smoking delayed ulcer healing. However, the mechanisms by which cigarette smoke depressed EGF production at the transcription level during biosynthesis are undefined and need further study.

Increased production of EGF in both salivary glands and gastric mucosa at the early phase of ulcer healing is critical. In the present study, both the salivary gland and mucosal EGF production were significantly ele-
Cigarette smoke exposure, which delayed ulcer healing at this early phase, also affected salivary gland and gastric mucosal EGF production as well as serum and mucosal EGF levels. Exogenous EGF (10 and 20 μg/kg iv) for the first 3 days completely reversed the delay of ulcer healing by cigarette smoking. However, it did not further accelerate ulcer healing, even if EGF was continuously given for a further 3 days, indicating that the reduction of EGF at the early phase of ulcer healing was critically related to the delay of ulcer healing induced by cigarette smoking. This result is in agreement with the findings of others that in acetic acid-induced gastric ulcer, administration of EGF (10 μg/kg, three times daily, sc) only accelerated the early phase of ulcer healing in sialoadenectomized rats (16). In the present study, the same dose of EGF had no ulcer healing effect in nonsmoking animals. Therefore, the beneficial effect of exogenous EGF on ulcer healing is probably a compensatory mechanism to replenish the deficiency of EGF in the cigarette smoking animals.

EGF exerts a variety of bioactivities. Both in vitro and in vivo studies have shown that EGF is a potent mitogenic factor that stimulates the synthesis of DNA, RNA, and proteins and therefore promotes cell proliferation (14). It also exerts an angiogenic effect (7, 23). Cigarette smoke exposure also significantly suppressed cell proliferation (Fig. 3) and angiogenesis in the gastric mucosa (Fig. 5). It is hypothesized that reduction of serum and mucosal EGF by cigarette smoking was associated with the inhibition of angiogenesis and mucosal cell proliferation. Indeed, supplementation of exogenous EGF before each cigarette smoke exposure significantly reversed the inhibitory effects of cigarette smoke exposure on angiogenesis in granulation tissues and mucosal cell proliferation at the ulcer margin (Figs. 3B and 5).

In addition, other factors such as NO and VEGF may well be involved in the regulation of angiogenesis (18, 36). It has been demonstrated that L-NAME, a NOS inhibitor, completely blocked the accelerating effect of EGF on ulcer healing, indicating that NO appeared to interact with EGF in the mechanisms of mucosal repair (3). EGF also showed the ability to stimulate VEGF synthesis in certain cells in vitro (27, 34). However, it is uncertain whether these mediators are also involved in the EGF-mediated angiogenesis during ulcer healing. It was confirmed that cigarette smoke exposure at 4% significantly decreased cNOS activity (Fig. 7), which was shown to be contributable to the depressive action of cigarette smoking on angiogenesis (19). Interestingly, exogenous EGF administration completely reversed the angiogenic action but not the effect on cNOS activity in the gastric mucosa, indicating that the reversal effect of EGF on angiogenesis was not mediated through the NO pathway. Furthermore, neither cigarette smoke exposure nor exogenous EGF affected mucosal VEGF level (Fig. 8), suggesting that VEGF was not associated with either the inhibition or reversal of angiogenesis induced by cigarette smoke exposure or exogenous EGF, respectively.

The discrepancy that EGF failed to stimulate VEGF synthesis in our in vivo model may be due to different doses of EGF used and different cells or tissues applied in these studies. The physiological role of gastric mucus in mucosal protection and ulcer healing has been well established. The polymeric structure of mucus gel becomes weaker and more easily digested during gastric ulceration (1, 5). Increased mucus and bicarbonate secretion at the site of injury provides a neutral pH environment that facilitates epithelial restitution (30). It is postulated that reduction of EGF in serum and gastric mucosa could subsequently affect gastric mucus content. Our results showed that the mucus content at the ulcer margin was significantly higher than that in the normal mucosa (Fig. 6). The increased gastric mucus not only protects the new proliferated cells from the digestion of acid and pepsin but also promotes the binding of growth factors to their receptors during the natural ulcer healing process. Cigarette smoke exposure at 4% significantly decreased the gastric mucus content 4 and 7 days after ulcer induction. Exogenous EGF completely reversed this inhibitory action by cigarette smoke (Fig. 6). These results indicate that reduction of EGF is involved in the impairment of mucus synthesis, which may also contribute to the delay of ulcer healing by cigarette smoking.

In summary, this study demonstrated for the first time that cigarette smoke delayed ulcer healing via a downregulation of EGF production in the salivary gland and gastric mucosa as a result of disruption of EGF mRNA transcription. This effect in turn significantly reduced angiogenesis, cell proliferation, and mucus synthesis in the gastric mucosa in the early phase of gastric ulcer healing. Apparently, deficiency of EGF is a critical event in delayed ulcer healing in smokers. In addition, both mitogenic and nonmitogenic actions of EGF are suggested to be involved in tissue repair in ulcerated stomachs.

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