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<tr>
<td><strong>Citation</strong></td>
<td>Gut, 2000, v. 47 n. 2, p. 170-177</td>
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<td><strong>Issued Date</strong></td>
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The role of polyamines in gastric mucus synthesis inhibited by cigarette smoke or its extract

L Ma, W P Wang, J Y C Chow, S K Lam, C H Cho

Abstract
Background—Cigarette smoking was shown to delay gastric ulcer healing and reduce synthesis of mucus, which is important for gastric ulcer protection and healing. Polyamines are important in these processes.

Aims—To study the effects of cigarette smoking on the synthesis of mucus and to investigate if such an effect is acting by interference with the polyamine pathway.

Methods—Gastric mucosal ornithine decarboxylase activity, mucus secreting layer thickness, and ulcer size were determined after different concentrations of cigarette smoke exposure (0, 2, or 4%) in intact animals and animals with ulcers. Synthesis of mucus and ornithine decarboxylase activity and mRNA expression were also assessed in cigarette smoke extract treated MKN-28 cells.

Results—Exposure to cigarette smoke significantly reduced the thickness of the mucus secreting layer and gastric mucosal ornithine decarboxylase activity in animals with or without ulcers. Spermidine not only reversed inhibition of mucus synthesis in both intact and ulcer bearing animals but also reversed the delay in ulcer healing. Cigarette smoke extract significantly reduced mucus synthesis and ornithine decarboxylase activity but not its mRNA expression in MKN-28 cells. The reduction in mucus synthesis was restored by spermidine.

Conclusions—Cigarette smoke and its extract repress mucus synthesis in vivo and in vitro, respectively. Reduction of ornithine decarboxylase activity in gastric mucosa is closely associated with this effect.

(Gut 2000;47:170–177)

Keywords: mucus; ornithine decarboxylase; cigarette smoking; ulcers

Epidemiological and animal studies have shown that cigarette smoking delays ulcer healing. However, the underlying mechanisms are not fully elucidated. The physiological role of mucus is important in mucosal protection and ulcer healing. Drugs which increase mucus synthesis accelerate recovery from ulceration.

Polyamines have been associated with cell proliferation during ulcer healing. A number of studies have demonstrated that polyamines are involved in epidermal growth factor (EGF) mediated gastroprotection, ulcer healing, and inhibition of acid secretion. Ornithine decarboxylase (ODC) is the first rate limiting enzyme for the biosynthesis of polyamines, including spermine, spermidine, and putrescine. Both its activity and mRNA expression were elevated by EGF. Furthermore, EGF has been demonstrated to stimulate gastric mucus synthesis. Therefore, we thought it would be interesting to determine if ODC and polyamines are also involved in gastric mucus synthesis.

A clinical study showed that smokers had a lower level of acid mucus substances in the gastric epithelium than non-smokers. However, because of the lack of an appropriate animal model for smoking, most studies have focused on the effect of nicotine on mucus synthesis and secretion. This compound is one of the major components of cigarette smoke and is easily accessible. Consequently, these studies reflect only part of the effects of cigarette smoking and cannot represent the action of smoking as a whole. Hence it would be useful to understand the exact relationship between cigarette smoking and mucus synthesis. We hypothesised that cigarette smoking might decrease mucus synthesis and secretion by interfering with the polyamine pathway, but little is known of the relationship between cigarette smoking and polyamine production or ODC activity in the gastric mucosa. Hence the aims of this study were to investigate: (1) how exposure to cigarette smoke affects mucus synthesis using a well established rat smoking model and the human gastric epithelial MKN-28 cell line; and (2) if ODC is involved in this process.

Abbreviations used in this paper: DFMO, DL-alpha-difluoromethylornithine; EGF, epidermal growth factor; MTI, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylterrazolium bromide; ODC, ornithine decarboxylase; PBS, phosphate buffered saline; TCA, trichloroacetic acid; PAS, periodic acid-Schiff; RTP-PCR, reverse transcription-polymerase chain reaction.
Materials and methods

CHEMICALS AND DRUGS
Chemicals and drugs were purchased from Sigma (St Louis, Missouri, USA) and organic solvents were purchased from Merck (Darmstadt, Germany), unless otherwise stated. In the animal study, spermidine was dissolved in distilled water and given orally at a dose of 100 mg/kg before each exposure to cigarette smoke.

ANIMALS AND INDUCTION OF GASTRIC KISSING ULCERS
The study was approved by the Committee on the Use of Live Animals for Teaching and Research in the University of Hong Kong. Male Sprague-Dawley rats (weighing 180–200 g) were reared on a standard laboratory diet (Ralston Purina Co., Chicago, Illinois, USA) and given tap water. They were kept in a room where temperature (22±1°C), humidity (65–70%), and day/night cycle (12 hours/12 hours) were controlled. Rats were deprived of food but had free access to tap water 24 hours before ulcer induction. Gastric kissing ulcers were produced by luminal application of an acetic acid solution, as described by Tsukimi and Okabe7 with some modifications.9 Thereafter, animals were fed a standard diet of laboratory chow and given tap water ad libitum.

CIGARETTE SMOKE EXPOSURE
Cigarette butts, without filters (R J Reynolds, Winston-Salem, North Carolina, USA) containing 1.2 mg of nicotine and 18 mg of tar per cigarette were used throughout the study. Intact or ulcerated rats (24 hours after ulcer induction) were exposed to different concentrations of cigarette smoke (0, 2, or 4%, vol/vol) in a chamber (39 cm×23.5 cm×21 cm) for one hour each day for several days. Procedures for exposure to cigarette smoke and the equipment used have been described previously.14 We have previously shown that this type of exposure does not affect the normal physiological functions of rats, such as acid/base balance, O2/CO2 in blood, heart rate, or blood pressure.14

Twenty four hours after the final exposure to cigarette smoke, rats were killed under ether anaesthesia by cervical dislocation. After washing with a cold saline solution, a longitudinal section of the stomach along the greater curvature (including the ulcer base and both sides of the ulcer margin in ulcerated stomachs) was obtained and fixed in 4% buffered formalin for 24 hours at 4°C for further histological study. The remaining glandular mucosa around the ulcer was scraped with a glass slide on an ice cold dish and immediately frozen in liquid nitrogen. Mucosal samples were stored at -70°C until assay.

EXTRACTION OF SUBSTANCES FROM CIGARETTE SMOKE
The substances in cigarette smoke were extracted using a smoke perfusion system. Briefly, smoke was forced through a series of bottles containing either 500 ml of chloroform (2x) or 95% ethanol (4x). The substances in smoke were thus absorbed in the solvents. The substances dissolved in ethanol were re-extracted with chloroform. All of the chloroform extractions were collected together and then concentrated by a RE 47 rotary evaporator (Yamato Scientific Co. Ltd, Tokyo, Japan) connected to a cooling system (Julabo F10, Labortherm GmbH, Seelbach, Germany). Our previous study showed that the chloroform extract but not the ethanol fraction potentiated ethanol induced gastric ulceration. Also, such fractions did not contain nicotine.20 The chloroform extract was subsequently used in examining the effect of cigarette smoke components on mucus synthesis in vitro.

DETERMINATION OF MUCUS SYNTHESIS IN MKN-28 CELLS
MKN-28 is a secretory type of human gastric carcinoma cell line derived from a moderately differentiated tubular adenocarcinoma.21 The rate of mucus synthesis was determined by measuring incorporation of D-[6-H] glucosamine into gastric mucosal glycoprotein according to the method of Takahashi and Okabe.22 Cells (1×10⁴) were incubated in 24 well culture plates for 16 hours; they were then washed twice with Ca²⁺/Mg²⁺ free phosphate buffered saline (PBS), followed by incubation with 0.5 ml of the medium containing [H]glucosamine HCl (Amersham, Little Chalfont, UK) in the presence of various substances (cigarette extract, polyamines, and DL-α-difluoromethylornithine (DFMO)), and/or corresponding vehicle at 37°C in 5% CO₂ for six hours. At the end of incubation, medium was aspirated and discarded. It was found that the amount of mucus glycoprotein secreted from cells into the medium was very low over the six hour incubation period. The remaining cells were washed twice with Ca²⁺/Mg²⁺ free PBS, solubilised with 0.4 ml of 0.3 mol/l NaOH, and neutralised with 0.4 ml of 0.3 mol/l HCl. The resulting aliquot was transferred to an Eppendorf tube. Then 0.5 ml of 50% trichloroacetic acid (TCA) was added to precipitate protein. Pellets were washed twice with 10% TCA, once with chloroform-methanol solution (1:1, vol: vol), and finally dried. These dried pellets were then dissolved in 25 µl of DMSO and the fractions were subjected to Sepharose CL-4B column chromatography for isolation of mucin, as described by Takahashi and Okabe.22 Radioactivity in the void fractions was measured as the amount of mucus synthesised by the cells. Mucus synthesis was expressed as [H] glucosamine incorporation (cpm) per 1×10⁴ cells.

ESTIMATION OF CELL VIABILITY
Cell viability was estimated using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT).23 MKN-28 cells (2×10⁴/100 µl medium) were seeded into a 96 well plate and incubated overnight for attachment. They were then incubated with cigarette extract, various drugs, and/or vehicle at 37 °C for six hours. At the end of incubation, the medium was aspirated. The remaining cells were incubated further with 0.25 mg/ml of MTT for three hours. MTT was extracted with 0.04 mol/l
HCl/isopropanol, and the colour change in the extract was measured at 595 nm. Cell viability was expressed as OD595 per 2x10⁴ cells.

DETERMINATION OF ORNITHINE DECARBOXYLASE (ODC) ACTIVITY IN THE GASTRIC MUCOSA AND MKN-28 CELLS

ODC activity was assessed by measuring the amount of ¹⁴CO₂ liberated from DL-[¹⁴C]ornithine.²⁴ Mucosal samples were collected as previously described and homogenised in 67 mmol/l sodium potassium phosphate buffer (pH 7.4). The homogenates were centrifuged at 20 000 g, 4°C, for 20 minutes. The superna-
tants were used for assay of enzyme activity. Cultured MKN-28 cells were scraped from the culture plate and placed in 10 mmol/l Tris HCl buffer (pH 7.4). The cells were then sonicated for 15 minutes under ice cold conditions and centrifuged at 2500 g, 4°C, for 10 minutes. Again the supernatants were used for the assay of ODC activity. A 300 µl aliquot of the superna-
tant was incubated in a stopped test tube in the presence of 2.5 mmol/l of L-[¹⁴C]ornithine for 15 minutes at 37°C. The ¹⁴CO₂, libera-
ted from the decarboxylation of ornithine was trapped by a piece of filter paper imregnated with 20 µl of 2.0 mol/l NaOH.

The paper was placed in a well connected to the stopper and suspended above the reaction mixture. The reaction was terminated by addition of 0.3 ml of 10% TCA. The radioactivity of ¹⁴CO₂ trapped in the filter paper was measured by a liquid scintillation counter (LS-
6500; Beckman Instruments, Fullerton, Cali-
ifornia, USA). Intra-assay and interassay coeffi-
cients of variation were 7.6% and 9.1%, re-
spectively. Protein content of the supernatant was measured by the Lowry method.²⁵ Enzyme activity was expressed as picomoles of ¹⁴CO₂ liberated per milligram of protein per hour.

DETERMINATION OF ODC mRNA BY RT-PCR

RT-PCR was used to semiquantitate mRNA for ODC. First, 4.0 µg of total RNA was used to generate the first strand of complementary DNA by using reverse transcriptase (Gibco BRL, Gaithersburg, Maryland, USA) following the manufacturer's instructions. PCR was performed for ODC and β-actin from the same complementary DNA samples using the follow-
ing primer pairs. For ODC, the sense primer (5'-CCTCAGTGAGCAATCCTC) which recognises a sequence in exon 7 and the antisense primer (5'-GCTGCACTAGGTT-
CCACGG) which recognises a sequence at the junction of exons 10 and 11 of human ODC cDNA were used.²⁶ For β-actin, the sense and antisense primers were 5' AAATGAGGACG

AGATCATGTTTGGAG and 5'-AGGGAGG

CAATGAT-CTTGATCCTT, respectively. PCR amplification was initiated using a commercial PCR Core Kit (Boehringer, Mannheim, Ger-
man). After the last cycle of amplification, sam-
ples were incubated for seven minutes at 72°C. PCR products (636 bp ODC fragment and 647 bp β-actin fragment) were visualised by UV illu-
mination after electrophoresis through 1% agar-
gose gels containing 0.5 µg/ml ethidium bromide. The gel photographs were submitted to a Multi-
Analyst (Bio-Rad Laboratories, Hercules, Cali-
ifornia, USA). Semiquantitative analysis of ODC mRNA was performed by comparing the inten-
sity of the PCR product of ODC to that of β-actin.

STATISTICAL ANALYSIS

Results are expressed as mean (SEM). Statisti-
cal analysis was performed using analysis of variance (ANOVA) and the unpaired Student's t test. p values less than 0.05 were considered statistically significant.

RESULTS

EFFECTS OF CIGARETTE SMOKE EXPOSURE ON THE MUCOUS SECRETING LAYER AND ODC ACTIVITY IN INTACT AND ULCERATED RATS

As shown in fig 1, the thickness of the gastric mucous secreting layer inside the mucosa in different measured fields was similar in intact rats. Exposure to cigarette smoke for one hour once daily for three, six, or nine days
ODC and gastric mucus in smoking

significantly decreased the thickness of the gastric mucous secreting layer in a dose dependent manner. In parallel, gastric mucosal ODC activity was also substantially reduced by exposure to cigarette smoke (fig 2A). In rats with ulcers, the thickness of the gastric mucous secreting layer four and seven days after ulcer induction was maximal at the ulcer margin, decreasing towards the distant normal area, and ultimately becoming similar to that of the normal mucosa (fig 3A, B). Exposure to cigarette smoke significantly reduced the thickness of the mucous secreting layer in all fields along the full length of the mucosal section (fig 3). Similarly, gastric mucosal ODC activity in ulcer bearing rats was significantly decreased by cigarette smoke exposure on days 4 and 7 after ulcer induction (fig 2B).
EFFECTS OF CIGARETTE SMOKE EXTRACT ON MUCUS SYNTHESIS, ODC ACTIVITY, AND mRNA EXPRESSION IN MKN-28 CELLS

The amount of $^3$H glucosamine incorporation in the mucin fraction was estimated as 75.71% of the whole cell preparation after Sepharose column chromatography. We used this column to determine mucus synthesis in the MKN-28 cell line. Incubation of MKN-28 cells with different concentrations of cigarette smoke extract (50, 100, or 200 $\mu$g/ml) for six hours significantly and dose dependently suppressed mucus synthesis, as indicated by a lower level of $^3$H glucosamine incorporation in the cultures exposed to smoke extract compared with the vehicle control (Fig 4). Similar results were found when the cells were incubated for the same period of time with DFMO, an ODC specific inhibitor (Fig 4). Further study demonstrated that cigarette smoke extract at concentrations of 100 and 200 $\mu$g/ml, which were sufficient to decrease mucus synthesis, also significantly reduced ODC activity in MKN-28 cells (Fig 5). However, ODC mRNA expression was not affected by cigarette smoke extract at either dose. The ratios of mRNA expression of ODC to $\beta$-actin in vehicle and smoke extract (100 and 200 $\mu$g/ml) groups were 0.80 (0.02), 0.81 (0.02), and 0.81 (0.02), respectively. Meanwhile, cell viability was unchanged by smoke extract or DFMO at the concentrations used in this study (Table 1A).

EFFECTS OF POLYAMINES ON MUCUS SYNTHESIS IN MKN-28 CELLS

Only spermidine at 0.1 mmol/ml showed a significant enhancing effect on mucus synthesis when MKN-28 cells were incubated with different polyamines for six hours. Putrescine and spermine at the concentrations used did not significantly affect mucus synthesis (Fig 6A). Thereafter, incubation of cells with spermidine (0.1 and 1 mmol/ml) in the presence of smoke extract (100 mg/ml) significantly reversed extract induced inhibition of mucus synthesis at the highest concentration of 1 mmol/ml (Fig 6B). Again, cell viability was unaffected by the polyamines at the concentrations used in this study (Table 1B, C).

EFFECTS OF SPERMIDINE ON THE MUCOUS SECRETING LAYER AND ULCER HEALING

In the animal study, it was found that cigarette smoke exposure at 4% for a one hour once daily for three days significantly reduced the thickness of the mucous secreting layer in both intact and ulcerated rats (Fig 7A, B). These findings support our previous in vitro study. Intragastric administration of spermidine (100 mg/kg) before each exposure to cigarette smoke completely reversed inhibition of the mucous secreting cell layer induced by cigarette smoke in both intact and ulcer bearing rats (Fig 7A, B) and significantly promoted ulcer healing in ulcerated animals (Fig 8). Interestingly, spermidine at the same dose did not affect either the mucous secreting layer or ulcer healing in non-smoking control animals.

Table 1  Effects of different forms of treatment on cell viability of MKN-28 cells

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<th>Treatment</th>
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<tbody>
<tr>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.57 (0.01)</td>
</tr>
<tr>
<td>Extract</td>
<td></td>
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<tr>
<td>50 $\mu$g/ml</td>
<td>0.55 (0.02)</td>
</tr>
<tr>
<td>100 $\mu$g/ml</td>
<td>0.57 (0.02)</td>
</tr>
<tr>
<td>200 $\mu$g/ml</td>
<td>0.54 (0.01)</td>
</tr>
<tr>
<td>DFMO</td>
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</tr>
<tr>
<td>10 mmol/ml</td>
<td>0.55 (0.01)</td>
</tr>
<tr>
<td>50 mmol/ml</td>
<td>0.56 (0.01)</td>
</tr>
<tr>
<td>B</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.55 (0.03)</td>
</tr>
<tr>
<td>Putrescine</td>
<td></td>
</tr>
<tr>
<td>0.1 mmol/ml</td>
<td>0.55 (0.01)</td>
</tr>
<tr>
<td>1 mmol/ml</td>
<td>0.59 (0.02)</td>
</tr>
<tr>
<td>Spermidine</td>
<td></td>
</tr>
<tr>
<td>0.01 mmol/ml</td>
<td>0.57 (0.02)</td>
</tr>
<tr>
<td>0.1 mmol/ml</td>
<td>0.55 (0.02)</td>
</tr>
<tr>
<td>1 mmol/ml</td>
<td>0.56 (0.02)</td>
</tr>
<tr>
<td>C</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.56 (0.03)</td>
</tr>
<tr>
<td>Extract</td>
<td></td>
</tr>
<tr>
<td>100 $\mu$g/ml</td>
<td>0.54 (0.02)</td>
</tr>
<tr>
<td>Extract+spermidine (0.1 mmol/ml)</td>
<td>0.54 (0.02)</td>
</tr>
<tr>
<td>Extract+spermidine (1 mmol/ml)</td>
<td>0.54 (0.02)</td>
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Values are mean (SEM) of six samples for each group.
Discussion
Cigarette smoking has been reported to increase both the incidence and recurrence of gastric and duodenal ulcers and also to delay ulcer healing. Our previous studies showed that both cigarette smoke and its extract derived from cigarette filters potenti- ated ethanol induced gastric mucosal damage and delayed the ulcer healing rate in rats. In the present study, cigarette smoke and its extract decreased the thickness of the mucus secreting layer inside the mucosa and also mucus synthesis in vitro, respectively (figs 1, 3, 4), indicating that modulation of gastric mucus may be part of the mechanism by which cigarette smoking intensifies ulceration and impairs ulcer healing. However, how cigarette smoking affects mucus synthesis is still unclear. Gastric mucus is secreted by epithelial cells and could be modulated by a number of factors secreted or synthesised locally in the gastric mucosa.

Polyamines are involved in many EGF mediated bioactivities including cell proliferation and acid secretion in the stomach. Sialo- adenectomised rats had reduced levels of gastric mucin indicating that salivary EGF is associated with gastric mucus synthesis. There is evidence that smokers have lower levels of salivary EGF than non-smokers, suggesting that salivary EGF production is impaired by cigarette smoking. Therefore, it would be help-
ful to determine if polyamine synthesis is affected by cigarette smoking and the role of polyamines in the regulation of gastric mucosal production. However, the relationships between cigarette smoking, polyamine production, and mucus synthesis in the gastric mucosa has never been investigated.

Using the present modified method to determine mucus synthesis in an isolated cell preparation, only spermidine showed a significant enhancing effect on mucus synthesis (fig 6A). This may be explained by the fact that different polyamines contributed differently to certain biochemical activities. Further study demonstrated that cigarette smoke extract decreased mucus synthesis, accompanied by a reduction in ODC activity in a dose dependent manner in MKN-28 cells (fig 4, 5). The former action was completely reversed by spermidine at a concentration of 1 mmol/l (fig 6B). DFMO, an ODC inhibitor, also induced a similar reduction in mucus synthesis (fig 4). These findings indicate that suppression of ODC activity is likely to be one of the mechanisms of inhibition of mucus synthesis induced by cigarette smoke extract. This effect was further supported by the in vitro study which showed that cigarette smoke exposure decreased the mucous secretory layer, accompanied by a reduction in mucosal ODC activity in both intact and ulcer bearing animals (fig 2). Oral administration of spermidine (100 mg/kg) markedly antagonised inhibition of the mucous secretory layer caused by cigarette smoke exposure (fig 7). Parallel to restoration of the mucous secretory layer by spermidine, the delay in ulcer healing was also substantially reversed (fig 8). Taken together, the in vitro and in vivo studies indicate that impairment of mucus synthesis as a result of suppression of mucosal ODC activity may contribute to the delay in ulcer healing induced by cigarette smoking. Interestingly, the thickness of the gastric mucous secreting layer after ulcer induction was substantially increased at the ulcer margin (figs 3, 7B). This may protect the newly proliferated cell against injury from the aggressive factors in the lumen. Furthermore, it may also provide the correct pH for epithelial cell restitution and binding of growth factors to their receptors at the ulcer margin. All of these actions could help in ulcer healing. Cigarette smoke exposure reduced the mucous secreting layer at the ulcer margin. This may be one of the mechanisms by which cigarette smoking delays ulcer healing. In the present study, ulcer induction itself significantly increased ODC activity in the gastric mucosa (fig 2). This is consistent with the finding that stress induced gastric mucosal damage activated ODC activity together with ulcer healing. Unlike mucosa with depressed ODC activity in smoking animals, supplementation of spermidine at the same dose did not have any effect on mucus synthesis in non-smoking animals.

The finding that cigarette smoke extract did not affect ODC mRNA expression in MKN-28 cells was surprising. Nevertheless, this suggests that the decrease in ODC activity was not caused by suppression of ODC gene activation. It is not known if translation to ODC protein, structural modification, or degradation was affected and further investigation is needed. However, it is known that mammalian ODCs are highly unstable. Their activities are dependent on many factors, including the low molecular weight thiols, pyridoxal phosphate. It has been suggested that in the absence of thiols, purified ODC polymerise through thiol disulphide interchanges and that polymerised forms have much less or no catalytic activity.

It is likely that cigarette smoke extract could impair ODC activity through interfering with the interaction between ODC and its cofactors. Our study showed that the smoke extract used in this study did not contain alkaloid, such as nicotine and yet markedly inhibited synthesis of mucus in gastric epithelial cells. This observation suggests that nicotine may not be the culprit for the acute adverse action of cigarette smoking on gastric ulceration.

In summary, although the present experimental conditions may not fully reflect the situation of active smoking, they are similar to passive smoking in humans. More importantly, we have demonstrated for the first time that short term exposure to cigarette smoke decreased mucosal ODC activity which subsequently impaired mucus synthesis. Administration of spermidine completely restored the inhibited gastric mucous synthesis and delayed ulcer healing. Because of the wide distribution of mucus in the gastrointestinal tract, and its pivotal role in protection and repair of the gastric mucosa, our findings may have substantial relevance in both basic and clinical settings. Hence our results have unveiled the possible mechanisms by which cigarette smoking adversely affects the gastrointestinal tract and may also have significant implications in the
treatment of ulcer disorders, especially those with a delayed healing rate, as well as other diseases where reduction of mucus secretion has been implicated.

The authors would like to thank the Hong Kong Research Grant Council for support and also Dr S Takahashi for advice on the determination of mucus synthesis in MKN-28 cells.

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