The etiology of ulcerative colitis (UC) remains unknown, although the risk of developing UC is apparently higher in non-smokers and ex-smokers. We have demonstrated in a colitis animal model that exposure to tobacco smoke could attenuate UC pathogenesis. The present study aimed to investigate and compare between the modes of action of nicotine and different fractions of tobacco smoke extract in the development of experimental colitis. The hapten 2,4-dinitrobenzene sulfonic acid (DNBS) was used to induce colitis in Sprague-Dawley rats. Results indicated that both tobacco smoke exposure and subcutaneous nicotine differentially reduced colonic lesion size, myeloperoxidase (MPO) activity, luminol-amplified free radical generation, and leukotriene B_4 formation in the inflamed colon of colitis animals. These phenomena were accompanied by the downregulation of colonic interleukin (IL)-1β and monocyte chemoattractant protein (MCP)-1 protein expression. By treating the colitis animals with various tobacco extracts, we further discovered that ethanol extract from filtered tobacco smoke could attenuate DNBS-evoked colonic damage and the elevated MPO activity, while at the same time it downregulated colonic IL-1β and MCP-1 protein expression. In contrast, the highest dose of the chloroform extract from the cigarette filter caused aggravating effects and overexpression of the pro-inflammatory cytokines and chemokines. These data suggest that effective attenuation of DNBS-induced colitis by tobacco smoke could be due to its nicotine content and possibly other flavonoid components found in the ethanol smoke extract.

**Key Words:** nicotine; tobacco smoke; neutrophils; chemotactic factors; colitis.

## INTRODUCTION

Ulcerative colitis (UC) and Crohn’s disease (CD) are dissimilar forms of inflammatory bowel disease (IBD) that have differential etiologies and unknown pathogenesis. One of the interesting but unexplained differences between UC and CD patients is the correlation with the incidence of cigarette smoking. The risk of developing UC was found to be greater in both ex-smokers and non-smokers, and the risk of developing CD was greater in active smokers (Harries et al., 1982; Kouroubakis et al., 1996). We also demonstrated the same phenomenon by using two distinct animal models that resemble human UC and CD, respectively (Guo et al., 1999; Ko et al., 2001). Nicotine, one of the major components of tobacco smoke, has been used as an alternative therapeutic agent for treating UC in some clinical trials (Guslandi and Tittobello, 1998; Sandborn et al., 1997). However, its low efficacy and the associated systemic side effects remain controversial (Kennedy, 1996). In fact, there are constituents in tobacco smoke other than nicotine that have been reported to possess anti-oxidative properties (Chen and Loo, 1995; Kamisaki et al., 1998). These include flavonoids, which are antioxidants and potent inhibitors of low-density lipoprotein oxidation (Bravo, 1998).

One of the pathogenic characteristics of IBD is the infiltration of neutrophils into intestinal tissues. When tissue influx of polymorphic nuclear cells (PMN) and macrophages occurs, a marked increase in the production of reactive oxygen metabolites (ROM) and leukotrienes (LT) will result as the secondary amplification of the inflammatory responses (Lauritsen et al., 1989). For this reason, new therapeutic agents derived for the treatment of IBD include inhibitors of neutrophil formation and activation. For instance, corticosteroids are capable of decreasing the margination of neutrophils, inhibiting neutrophil aggregation and interfering with neutrophil eicosanoid metabolism (Claman, 1984). This could explain why neutrophilic chemotactic activity was found to be higher in UC mucosa than in normal mucosa. 5-Lipoxygenase (5-LOX), primarily localized in leukocytes, is responsible for the synthesis of LT and other products that have played important roles in tissue inflammation. As a result, most of the chemotactic activities were blocked by anti-LTB_4 antibody as demonstrated in some *in vitro* studies, suggesting that LTB_4 could be one of the major chemotactic factors in UC (Lobos et al., 1987). Moreover, there have been several reports showing that LTB_4 is responsible for the pathophysiology in...
both human and rat colitis models (Nakamaru et al., 1994; Schmidt et al., 1995; Zhou and Mineshita, 1999). In fact, an increased colonic level of both LTB₄ and luminol-amplified ROM was documented in patients with active IBD (Mahida et al., 1989; Suematsu et al., 1987). In a clinical study, dietary supplementation of fish oil in patients with IBD resulted in the reduction of rectal dialysate LTB₄ level and improvement in UC disease markers (Stenson et al., 1992).

Studies have consistently revealed that chemokines are upregulated in the colonic tissues of IBD patients (Banks et al., 2003). One of the specific examples is that the percentage of cells expressing monocyte chemoattractant protein (MCP)-1 was found to be significantly enhanced in all colonic biopsies from active UC patients, when compared with the data from healthy individuals (Ugucioni et al., 1999). In turn, the upregulated chemokine expression appeared to correlate with increased disease activities (Banks et al., 2003), which could be related to the spontaneous release of pro-inflammatory cytokines from the inflamed intestinal mucosa (Reimund et al., 1996). Among these, tumor necrosis factor (TNF)-α was significantly increased only in the colonic biopsies from CD patients, but not in UC patients (McCormack et al., 2001). It is therefore of great interest to determine the involvement of other major pro-inflammatory cytokines such as interleukin (IL)-1β and the associated chemokine upregulation during UC development and after chemotherapy.

In the present study, the effects of unfiltered or filtered tobacco smoke, nicotine, and various extracted tobacco smoke components in experimental UC development were explored in a rat colitis model. Moreover, the involvement of various chemotactic factors in these processes was also investigated.

**MATERIALS AND METHODS**

**Animals.** Male Sprague-Dawley rats (180–200 g) were kept in a room with controlled temperature (22 ± 1°C), humidity (55–60%) and 12-h light–dark cycles, and were acclimatized for at least 2 weeks prior to experimentation. The animals had free access to standard laboratory chow (Ralston Purina, USA) and tap water ad libitum. The experimental procedures were approved by our institutional animal research ethics committee with reference to the European Community Guidelines for the Use of Experimental Animals.

**Induction of colitis and tissue biopsy.** 2,4-Dinitrobenzene sulfonic acid (DNBS), a hapten molecule, was used to induce experimental colitis in rats (Ko et al., 2001). Histopathological observation had proven that DNBS-induced damage resembles human UC colitis (Hawkins et al., 1997). Nevertheless, in studying chronic UC, other hapten models such as the dextran sulfate sodium (DSS) method could be employed instead (Elson et al., 1995). Intracolonic administration of DNBS (30 mg in 250 µl of 50% ethanol) was accomplished by inserting a siliconized rubber cannula intrarectally into a ketamine/xylazine-anesthetized rat such that the tip was 8 cm proximal to the anus. All experimental animals were sacrificed 4 days after colitis induction. We had previously demonstrated that colonic inflammation began to emerge 1 h after intracolonic DNBS administration, and that hemorrhagic lesions and ulcers were visualized in the distal colon by 6 h. The choice of 4 days after colitis induction as the experimental endpoint designates the beginning of active wound healing, which is susceptible to drug intervention. After macroscopic lesion areas were assessed, biopsies were taken from the distal colonic region at 2–8 cm proximal to the anus and stored at −85°C for various assays and Western analyses.

**Treatment with tobacco smoke and nicotine.** The passive smoking method employed in this study was modified from the original design by Chow and Cho (1996) and was further discussed in Guo et al. (1999). Rats were exposed to a fixed concentration (4% v/v) of smoke from commercial cigarettes (Kings, UK), with a nicotine content of 1.1 mg/cigarette and a tar content of 15 mg/cigarette, in a ventilated smoking chamber (39 × 23.5 × 21 cm³). A lighted cigarette with the mouthpiece filter removed (by cutting the wrapping paper circumferentially at the point where the glass-fiber filter meets the tobacco leaves) was plugged into a home-made glass mouthpiece attached to a 3-way stopcock. To maintain a constant 4% v/v smoke/air concentration inside the chamber, two peristaltic pumps (Masterflex, Cole-Parmer Instrument Co., Vernon Hills, IL) were used to deliver fresh tobacco smoke (at 40 ml/min) and fresh air (at 960 ml/min) simultaneously. Control rats were subject to the same procedures, except that they received only fresh air from the two pumps. The duration of smoke exposure (or pure air in the control group) was 1 h daily for 3 consecutive days (days 2–4), commencing 24 h after enema DNBS administration on day 1. The experiment was then repeated using cigarettes with intact mouthpiece filter attached (in the filtered smoke group). To ensure a consistent supply of tobacco smoke to the chamber during both smoking sessions with non-filtered or filtered smoke, the cigarette would be replaced by a new one when there was a 5-mm unburned length (with tobacco leaves) left in the glass mouthpiece. In addition, the whole set-up for the smoking experiments was placed inside a chemical fume hood. This smoking method was proved not to affect the blood pH and O₂/CO₂ balance in the animals based on blood gas data (pH 7.43, 32.14–32.88 mm Hg pCO₂ and 91.21–91.28 mm Hg pO₂). The mean serum concentration of nicotine in rats’ blood collected 45 min after a 1-h exposure to 4% v/v tobacco smoke was measured at 0.136 ng/µl with non-filtered smoke and 0.128 ng/µl with filtered smoke.

Alternatively, nicotine (Sigma, St. Louis, MO) dissolved in normal saline was injected subcutaneously into rats after enema DNBS administration by three regimens, as follows: a single dose of 4 mg/kg on day 2; a single dose of 8 mg/kg on day 2; multiple doses of 4 mg/kg on three consecutive days from day 2 to day 4.

**Extraction of filtered tobacco smoke and cigarette filter.** Extracts of either the filtered smoke or the used cigarette filter were obtained according to methods described previously (Chow et al., 1997; Ma et al., 2000). The constituents in tobacco smoke were extracted by a perfusion system including four bottles of 96% ethanol and two bottles of chloroform with the flow rate of 700 ml/min using a peristaltic pump. The extraction process and different extracts obtained are summarized in Figure 1. First, puffs of smoke from cigarettes with filters attached were allowed to sieve through the filter and pass into ethanol and chloroform for extraction, resulting in the collection of the ethanol extract of filtered smoke (FSEE) and the chloroform extract of filtered smoke (FSCE), respectively. The used filters of the cigarettes were then soaked in 5% hydrochloric acid for 1 h with sonication. The resulting solution was mixed with chloroform, followed by separation from the acidic aqueous layer (forming the first chloroform extract of filter, FCE1). Sodium hydroxide was added to the aqueous layer to a pH of 10. Chloroform extraction was repeated once again to obtain the second chloroform extract of the filter (FCE2). All the extracted products were concentrated to form different extracts by using a rotary evaporator connected to a cooling system (Buchi, Germany), followed by lyophilization (Labconco, Kansas City, MO).

**Treatment with tobacco extracts.** Various tobacco smoke or filter extracts suspended in Tween 80 (Sigma, St. Louis, MO) were injected into different groups rats intraperitoneally daily for 3 consecutive days after colitis induction, with a similar schedule as that used for tobacco smoke exposure. The doses used were 5, 10, or 20 mg/kg for FSEE, 2.5, 5, or 10 mg/kg for FCE1 and 0.68, 1.36, or 2.72 mg/kg for FCE2, respectively. The median doses of the extracts used (10 mg/kg for FSEE, 5 mg/kg for FCE1, and 1.36 mg/kg for FCE2) were calculated based on the experiment using 4% v/v of tobacco smoke (Chow...
of the reaction product 2,2'-diaminobenzidine (DAB) under pH 5.0. The final values were represented as count per minute.

**Assessment of ROM in colonic tissue.** Reactive oxygen metabolite production in the excised colonic tissues can be measured by a luminol-amplified chemiluminescence assay as described by Simmons and co-workers (1992). Luminol was dissolved in Dulbecco’s phosphate-buffered saline (PBS) at a concentration of 300 μmol/l with added glucose (5 mmol/l) on the day of experiment. Luminol can react with ROM to form 3-aminophthalate. The excited electrons in this compound reverted to their ground state with the emission of energy as light (chemiluminescence), which was detected by the photomultiplier tubes of a scintillation counter (Beckman LS6500, Ramsey, MN). The final value of each colonic sample was represented as count per minute.

**Analysis of IL-1β and MCP-1 protein expressions in colonic tissue.** To further explore the contribution of other leukocytic chemotactic factors in the pathogenesis of experimental colitis as well as in the actions of nicotine and tobacco smoke components, protein expression of the pro-inflammatory cytokine IL-1β and of the CC chemokine MCP-1 in the colonic tissues was determined by Western blot analysis (Bless et al., 2000; Di Loreto et al., 2004). Colonic tissues were weighed and homogenized for 30 s at 4°C in lysis buffer (50 mM Tris, 150 mM NaCl, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2 mM EDTA, 1% Triton X-100, and 10% glycerol) with 1 mM phenylmethanesulfonyl fluoride, aprotinin (5 mg/ml), and pepstatin A (5 mg/ml) added freshly. The colonic tissue samples were then centrifuged at 13,000 × g for 20 min and the resulting supernatant was collected. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out on the protein samples (100 μg). The separated proteins were transferred to a nitrocellulose membrane, which was then blocked in TBST (0.2 M Tris, 1.37 M NaCl, 1 N HCl, and 0.1% Tween 20) with 5% non-fat dry milk for 1.5 h at room temperature. The blotted membrane was then incubated with the respective primary antibodies (anti-IL-1β or anti-MCP-1) or actin (as internal control) overnight at 4°C, and subsequently incubated in secondary antibody for 1 h at room temperature. Upon incubation in the ECL Western blotting detection reagent (Amersham, USA) for 1 min, the IL-1β and MCP-1 bands were visualized by autoradiography on x-ray film (Fuji, Japan). Quantification of individual band density was carried out by a video densitometer (Scan Maker III, Microtek, Taiwan, ROC) expressed as volume count × mm², which was normalized by using actin as internal control.

**Statistical analysis.** Results were expressed as mean ± SE. Differences between two groups were examined using one-way analysis of variance (ANOVA) with various post-hoc tests. Dunnett’s test was first performed to compare between the effects of the individual treatment group (or the normal group) with the respective control. Additional analysis was performed by Duncan’s new multiple-range test, with which statistical differences can be found with smaller mean differences (e.g., between the non-filtered smoking group and the control). Moreover, the Newman-Keuls procedure was conducted to compare between the magnitude of effects between different treatment groups. Finally, the Pearson correlation coefficient test was conducted to determine if there were any dose–response relations in the effects of various extracts from tobacco smoke and filter. A p value of less than 0.05 was considered significantly different.

**RESULTS**

**Alleviation of DNBS-Induced Colitis by Tobacco Smoke and Nicotine**

Enema DNBS caused lesion formation and severe inflammation in the distal colon of control rats. The pathogenic features were associated with tissue edema, represented by the colonic edema index (expressed as the colonic weight per unit body weight; data not shown), along with shortening of the inflamed colon. Daily exposure of non-filtered tobacco smoke...
following colitis induction significantly reduced the area of inflammation and active ulceration in the colitis animals (Fig. 2), as well as alleviating the associated pathogenic conditions. Owing to the fact that some detrimental compounds such as hydrocarbons and terpenoids could be trapped in the cigarette’s filter, while potentially colitis-ameliorating compounds like nicotine and other alkaloids could pass through (Chow et al., 1997), we exposed the experimental animals to filtered tobacco smoke so as to determine whether there would be a further improvement in lesion and inflammation development. On the one hand, results had shown that the differences in anti-inflammatory effects and diminution of lesion size between the non-filtered and filtered tobacco smoke groups were not reaching a statistically significant level. On the other hand, subcutaneous nicotine also demonstrated significant and similar anti-inflammatory and lesion-alleviating effects with either a single dose of 8 mg/kg or multiple doses of 4 mg/kg nicotine, whereas a single dose of 4 mg/kg nicotine had no effect (Fig. 2). There was no significant difference between the magnitudes of anti-lesion actions in various effective treatment groups (Newman-Keuls analysis).

**Attenuation of the Exaggerated Neutrophil Recruitment and Free Radical Formation**

Myeloperoxidase activity is a marker for neutrophil activation, which signifies the degree of local tissue inflammation. In this study, colonic MPO activity was dramatically elevated by more than threefolds in the inflamed colon of control rats, when compared to the intact colonic tissue of non-diseased animals (Normal). Exposure to non-filtered smoke, filtered smoke, and the two regimens of nicotine treatment (4 mg/kg × 3 and 8 mg/kg × 1) all significantly decreased the DNBS-activated MPO activity (Fig. 3), a finding that was in line with the relative degrees of amelioration in colonic lesion formation (Fig. 2). Again, there was no significant difference between the effects on MPO activity induced by various effective treatment groups (Newman-Keuls analysis). Alternatively, the mucosal level of LTB₄, a neutrophil chemotactic factor, was also drastically increased in the control colitic tissues when compared with its basal level in normal colonic tissues (Fig. 4). Filtered tobacco smoke exposure and nicotine regimens caused marked inhibition of such elevation in colonic LTB₄ content. Nonetheless, although non-filtered tobacco smoke had a tendency to produce similar inhibitory action on LTB₄ production, the effect did not arrive at a statistically significant level after performance of two different post-hoc (Dunnett’s and Duncan’s) tests (Fig. 4).

Luminol-amplified chemiluminescence assay can be used to detect free radicals generated predominantly via a MPO-catalyzed reaction (Hawkins et al., 1997). Only a negligible amount of luminol-amplified chemiluminescence was observed in the excised colonic tissues of normal animals. Our findings also showed that the vast amount of MPO-derived free radicals found in the inflamed colonic tissue was significantly decreased after treatment with either filtered tobacco smoke or nicotine.
regimens (Fig. 5), much like their respective inhibitory actions on LTB₄ production (Fig. 4). When compared with the inhibitory effect on MPO activity, non-filtered tobacco smoke exposure did not significantly alleviate MPO-derived free radical formation, as in the case of LTB₄ level (Dunnett’s and Duncan’s tests).

Modulation of the Protein Expression of IL-1β and MCP-1 in the Inflamed Colonic Tissue

Report from animal colitis model had demonstrated significant elevation in mRNA expression of the cytokines IL-1β and IL-6, and of the chemokine macrophage inflammatory protein-1α and MCP-1, which could persist for 2 weeks (Sun et al., 2001). In fact, MCP-1 activates macrophages and increases the migration of monocytes into tissue during inflammation, whereas IL-1β upregulates constitutive MCP-1 mRNA level in colonic cell culture (Reinecker et al., 1995). Figures 6 and 7 illustrate that colonic IL-1β and MCP-1 proteins were both overexpressed in colitis tissues, and the overexpression was concomitantly attenuated by filtered cigarette smoke exposure and nicotine regimens. However, no significant difference was found between the modulation of protein expression by various treatment groups (Newman-Keuls analysis). Representative bands of IL-1β, MCP-1, and actin for each experimental group are shown in Figure 12A.

Effects of Ethanol Extract of Filtered Tobacco Smoke and Filter Chloroform Extract 1 and 2 on DNBS-Induced Colitis and Neutrophil Activation

Ethanol and chloroform extracts from tobacco smoke were analyzed for their chemical types by thin-layer chromatography

![FIG. 4.](image-url) Exposure to filtered tobacco smoke as well as different regimens of nicotine attenuate the elevated colonic LTB₄ level in colitis rats. Treatment regimens were the same as those stated in Figure 2. The LTB₄ level was determined by an enzyme immunoassay method as described in Materials and Methods. The final values of LTB₄ level were represented as picograms per milligram of colonic tissue. Data represent the means ± SE of seven animals. A pound sign (#) indicates a significant difference between the group of normal animals without colitis and the DNBS colitis control group (# p < 0.01). An asterisk (*) indicates a significant difference between a treatment group and the DNBS control group (* p < 0.05).

![FIG. 5.](image-url) Exposure to filtered tobacco smoke as well as different regimens of nicotine alleviate MPO-derived free radical formation in colitis rats. Treatment regimens were the same as those stated in Figure 2. Myeloperoxidase-derived free radical formation was determined by a luminol-amplified chemiluminescence assay as described in Materials and Methods. Reactive oxygen metabolite was quantified by the photomultiplier tubes of a scintillation counter and the final values were represented as count per minute (× 10³ cpm). Data represent the means ± SE of seven animals. A pound sign (#) indicates a significant difference between the group of normal animals without colitis and the DNBS colitis control group (#p < 0.001). An asterisk (*) indicates a significant difference between a treatment group and the DNBS control group (*p < 0.05, **p < 0.01).

![FIG. 6.](image-url) Exposure to filtered tobacco smoke as well as different regimens of nicotine attenuate the upregulation of colonic IL-1β protein expression in colitis rats. Treatment regimens were the same as those stated in Figure 2. Western blot analysis with specific antibody against IL-1β and actin (internal control) was performed. Colonic tissue extracts were obtained as described in Materials and Methods. Quantification of individual band density was carried out by a video densitometer and was normalized by the actin band. Data indicate the means ± SE of the ratio of IL-1β to actin band density from the blots of colonic tissue extract of seven animals. A pound sign (#) indicates a significant difference between the group of normal animals without colitis and the DNBS control group (#p < 0.001). An asterisk (*) indicates a significant difference between a treatment group and the DNBS control group (*p < 0.01).
and gas chromatography/mass spectrophotometry. The chemicals identified in FSEE were mainly alkaloids, including nicotine, whereas terpenoids, phenolic compounds, hydrocarbons, organic acids, fatty acids, and flavonoids were found in FCE1, with no alkaloid being observed (Chow et al., 1997). Furthermore, the acidic aqueous layer obtained from FCE2 contained a trace amount of alkaloid, which is much lower than those found in the FSEE fraction from filtered smoke.

Intraperitoneal administration of the two higher doses of FSEE (10 and 20 mg/kg) significantly reduced DNBS-induced colonic lesion formation (Fig. 8, top), a finding that was in concert with their ameliorating potential on the elevated MPO activity (Fig. 9, top). In contrast, intraperitoneal administration of the highest dose of FCE1 (10 mg/kg) significantly aggravated DNBS-induced colonic damage (Fig. 8, bottom) with concurrent potentiation of the elevated colonic MPO activity (Fig. 9, bottom). But there was no dose–response relation in the effect of FSEE and FCE1 (Pearson correlation coefficient test). Treatment with FCE2 caused no effect in either colitis development or in modulating colonic MPO activity.

Effects of Ethanol Extract of Filtered Tobacco Smoke and Filter Chloroform Extract 1 and 2 on Colonic Protein Expression of IL-1β and MCP-1

We further delineated the effects of various tobacco extracts on colonic protein expression of IL-1β and MCP-1. Our results had shown that treatment with all three doses of FSEE induced a downregulation of both IL-1β and MCP-1 protein expression in colonic tissues (Fig. 8, top; Fig. 9, top), a result that was somewhat consistent with their anti-inflammatory actions (Fig. 8, top; Fig. 9, top) and that had effects similar to those demonstrated by filtered tobacco smoke and nicotine treatments (Fig. 6 and 7). In contrast, treatment with FCE1 (10 mg/kg) caused an upregulation of colonic IL-1β and MCP-1 protein expression (Fig. 10, bottom; Fig. 11, bottom), which possessed a direct correlation with their pro-inflammatory actions in the colon (Fig. 8, bottom; Fig. 9, bottom). Again, no dose–response relation was found in the effect of FSEE and FCE1 (Pearson correlation coefficient test). Representative bands of IL-1β, MCP-1, and actin for each experimental group are shown in Figure 12B and 12C.

FIG. 7. Exposure to filtered tobacco smoke as well as different regimens of nicotine attenuate the upregulation of colonic MCP-1 protein expression in colitis rats. Treatment regimens were the same as those stated in Figure 2. Western blot analysis with specific antibody against MCP-1 and actin (internal control) was performed. Colonic tissue extracts were obtained as described in Materials and Methods. Quantification of individual band density was carried out with a video densitometer and was normalized by the actin band. Data indicate the means ± SE of the ratio of MCP-1 to actin band density from the blots of colonic tissue extract of seven animals. A pound sign (†) indicates a significant difference between the group of normal animals without colitis and the DNBS colitis control group (†p < 0.001). An asterisk (*) indicates a significant difference between a treatment group and the DNBS control group (*p < 0.01, **p < 0.001).

FIG. 8. Ethanol extract of filtered tobacco smoke (FSEE) reduces the area of colonic damage, whereas chloroform extract 1 of the filter (FCE1) aggravates colonic injury in colitis rats. Enema DNBS was administered to rats to induce experimental colitis. (Top) FSEE (5, 10, or 20 mg/kg) and (bottom) FCE1 (2.5, 5, or 10 mg/kg) were given intraperitoneally to the rats daily for 3 consecutive days after colitis induction. All animals were sacrificed 4 days after colitis induction. Colonic lesion area was measured in mm². Data represent the means ± SE of seven animals. An asterisk (*) indicates a significant difference between a treatment group and the DNBS control group (*p < 0.05, **p < 0.01).
DISCUSSION

The strong association between smoking and IBD development has been known for many years. We are the first group to exemplify the anti-inflammatory and immunomodulating effects of tobacco smoke on experimental colitis (Ko et al., 2001). In the present study, exposure to non-filtered and filtered tobacco smoke reduced the severity of colonic inflammation and lesion formation induced by DNBS. Because the anti-inflammatory action of tobacco smoke involved reduction of the elevated colonic MPO activity, prevention of neutrophil activation and infiltration into the inflamed tissues could be important. LTB4 is one of the most potent chemotactic and chemokinetic metabolites of arachidonic acid, and it plays a permissive role in the action of neutrophils during the amplification of intestinal inflammatory reactions, including colitis (Nielsen and Rask-Madsen, 1996). As a result, lowering of the LTB4 level could attenuate neutrophil activation and infiltration into the inflamed colonic tissue, and consequently free radicals generated by activated neutrophils and other granulocytes would also be markedly reduced. Our earlier findings had indicated a significant decline in colonic LTB4 level and inhibition of ROM production by both nicotine and filtered tobacco smoke treatments. Nicotine was previously shown to decrease LTB4 production from rat alveolar macrophages (Sugiyama et al., 1989). Besides, it was also discovered that nicotine could enhance neutrophil sequestration, possibly by scavenging other oxidants in tobacco smoke (Aoshiba et al., 1994). Other proposed protective mechanisms of nicotine on UC include the boosting of mucin synthesis on colonic luminal surface, release of nitric oxide that counteracts with eicosanoid metabolism, and the increase in intestinal trefoil factors.

FIG. 9. An ethanol extract of filtered tobacco smoke (FSEE) attenuates the activated colonic MPO activity in colitis rats, whereas chloroform extract 1 of the filter (FCE1) intensifies the activated colonic MPO activity in colitis rats. Treatment regimens were the same as those stated in Figure 8. Myeloperoxidase activity was determined by a modified tetramethylbenzidine method as described in Materials and Methods. The final values of MPO activity were represented as units per gram of colonic tissue. Data represent the means ± SE of seven animals. A pound sign (#) indicates a significant difference between the group of normal animals without colitis and the DNBS colitis control group (#p < 0.01). An asterisk (*) indicates a significant difference between a treatment group and the DNBS control group (*p < 0.05, **p < 0.01, ***p < 0.001).

FIG. 10. Ethanol extract of filtered tobacco smoke (FSEE) attenuates while the chloroform extract 1 of the filter (FCE1) intensifies the activated upregulation of colonic IL-1β protein expression in colitis rats. Treatment regimens were the same as those stated in Figure 8. Western blot analysis with specific antibody against IL-1β and actin (internal control) was performed. Colonic tissue extracts were obtained as described in Materials and Methods. Quantification of individual band density was carried out by a video densitometer and was normalized by the actin band. Data indicate the means ± SE of the ratio of IL-1β to actin band density from the blots of colonic tissue extract of seven animals. A pound sign (#) indicates a significant difference between the group of normal animals without colitis and the DNBS colitis control group (#p < 0.01, ##p < 0.001). An asterisk (*) indicates a significant difference between a treatment group and the DNBS control group (*p < 0.05, **p < 0.01).
that eventually improve mucosal restitution (Wu and Cho, 2004). Although many clinical trials have suggested that nicotine may not be effective in mono-therapy, evidence from a 12-month study indicated that nicotine-induced remission of UC could last longer than that obtained by conventional therapeutic agents such as oral corticosteroids (Guslandi, 1999). In addition, this report had also shown that 6 mg of both oral and transdermal nicotine could be well tolerated in UC patients, and this was thus thought to be the highest therapeutic dose with a low risk of adverse effects in the human body (Green et al., 1999; Ingram et al., 2004).

Tobacco smoke could result in a comparable serum concentration of nicotine to the level in animals treated with non-filtered smoke (Chow et al., 1997), a level much lower than that achieved after nicotine treatments. Under such circumstances, because tobacco smoke was capable of producing similar protection against colitis development, as in the nicotine-treated animals, there must be some unknown substance(s) in the tobacco smoke that could play a permissive role in the amelioration of colitis. However, this hypothesis can only be clarified by examining individual components of the tobacco smoke. It should be noted that the cigarette filter can separate the tar and gas phases of tobacco smoke. Most of the particles in the tar phase, including ROS, hydrocarbons, and phenols, are retained in the filter (Pryor and Stone, 1993). Our previous studies also revealed that extracted compounds from the tar phase of tobacco smoke could repress mucus synthesis in vivo and in vitro by inhibiting polyamine synthesis (Ma et al., 2000) and thus potentiate ethanol-induced gastric mucosal injury (Chow et al., 1997). In accordance with this finding, the

FIG. 11. Ethanol extract of filtered tobacco smoke (FSEE) attenuates while the chloroform extract 1 of the filter (FCE1) intensifies the activated upregulation of colonic MCP-1 protein expression in colitis rats. Treatment regimens were the same as those stated in Figure 8. Western blot analysis with specific antibody against MCP-1 and actin (internal control) was performed. Colonic tissue extracts were obtained as described in Materials and Methods. Quantification of individual band density was carried out with a video densitometer and was normalized by the actin band. Data indicate the means ± SE of the ratio of MCP-1 to actin band density from the blots of colonic tissue extract of seven animals. A pound sign (#) indicates a significant difference between the group of normal animals without colitis and the DNBS colitis control group (#p < 0.01, ##p < 0.001). An asterisk (*) indicates a significant difference between a treatment group and the DNBS control group (*p < 0.05).

FIG. 12. Representative bands of IL-1β, MCP-1, and actin after different treatments. (A) N = normal, C = control, FS = multiple (3×) treatments of 4% v/v filtered tobacco smoke, Nic(4 × 3) = multiple (3×) treatments of 4 mg/kg nicotine, Nic(8) = single treatment of 8 mg/kg nicotine. (B) N = normal, C = control, FSEE(5) = multiple (3×) treatments of 5 mg/kg FSEE, FSEE(10) = multiple (3×) treatments of 10 mg/kg FSEE, FSEE(20) = multiple (3×) treatments of 20 mg/kg FSEE. (C) N = normal, C = control, FCE1(2.5) = multiple (3×) treatments of 2.5 mg/kg FCE1, FCE1(5) = multiple (3×) treatments of 5 mg/kg FCE1, FSEE(10) = multiple (3×) treatments of 10 mg/kg FCE1.
detrimental compounds may aggravate colitis formation and also antagonize the effects of other protective agents. Although the gas phase of tobacco smoke also contains radicals such as nitric oxide and reactive olefins, these organic radicals usually have a shorter half-life (typically less than 1 s) than those contained in tar phase smoke (Pryor and Stone, 1993). Hence, most of the alkaloids in tobacco smoke, including nicotine, that pass through the filter could produce prominent anti-oxidative effects that contribute to colonic protection in a similar manner to the action of systemic nicotine treatment alone.

To clarify the differential effects of filtered and non-filtered smoke, we performed ethanol and chloroform extractions to collect the chemical components of gas phase smoke and tar phase smoke, respectively. The ethanol extract of filtered smoke (FSEE) was shown to be protective against colitis formation, whereas the administration of FCE1 at its highest dose (10 mg/kg) was detrimental to colitis development. Ma et al. (2000) reported that the chloroform extract of tobacco smoke contains terpenoids and phenolic compounds. These results actually supported our previous suggestion that the beneficial effects of filtered smoke and nicotine were more prominent than those of non-filtered smoke because of the presence of damaging factor(s) in the latter. We also examined the effect of FCE2 using the same colitis model, but there was no significant effect observed (data not shown). These findings indicate that the quantity of detrimental substances contained in filtered smoke was not high enough to worsen colitis development, while the content of potential protective mediators trapped in the filter was also too minimal to cause any effect.

Polymorphonuclear leukocytes migrate in response to chemotactic factors, which can be activated to produce certain chemokines. Neutrophils and macrophages in inflamed intestine of IBD patients synthesize and secrete large amounts of chemokines (MacDermott, 1999). Nonetheless, epithelial chemokine production could be an important target in the therapy of IBD (van Deventer, 1997). Our findings actually demonstrated that there was concomitant inhibition of colonic MPO activity and downregulation of MCP-1 protein expression by FCE1 extract, and nicotine. The expression of the MCP-1 gene in vessel-associated cells may indicate its involvement in regulating the adhesion of blood monocytes to endothelial cells (Mazzucchelli et al., 1996). Although monocyte recruitment is the primary biological role of MCP-1, it was found that MCP-1 is also capable of attracting neutrophils via the production of LTB4 (Matsukawa et al., 1999). In contrast, LTB4 could reversibly induce MCP-1 production during active inflammation. In addition, findings from other studies have suggested that MCP-1 depletion inhibits neutrophil influx by downregulating LTB4 production in intestinal tissues (Crooks and Stockley, 1998), which resembles the situation in colitis animals treated with filtered cigarette smoke, FSEE extract, or nicotine.

Along with chemokine activation, the expression of most pro-inflammatory cytokines, notably IL-1, IL-6, and TNF-α, is markedly enhanced in the intestinal mucosa of IBD patients (Kmiec, 1998). Some protective agents could act by reducing both colonic LTB4 and IL-1β levels, which would consequently improve the state of oxidative stress in the colon (Galvez et al., 2001). Our findings on the effects of filtered cigarette smoke, FSEE extract, and nicotine on colonic IL-1β expression in fact reflected the phenomenon of a clinical study on smokers with UC, which had shown a significant reduction in IL-1β level in the patients’ colonic mucosa (Sher et al., 1999). In spite of the report that the Th-1 cytokine IL-1β could modulate LTB4 production from monocytes (Montero et al., 2000), other groups have stated that synthesis of such cytokines is in turn determined by LTB4 level in the inflammatory tissues (He et al., 2002; Kuwabara et al., 2000). Moreover, the inhibition of IL-1β production also seemed to be associated with the downregulation of MCP-1 protein synthesis in the colonic mucosa. Nonetheless, a similar correlation between MCP-1 and another pro-inflammatory cytokine, TNF-α, seems to occur in CD patients only (McCormack et al., 2001). According to the increased colonic protein expression of both MCP-1 and IL-1β induced by administering the highest dose of FCE1 extract, it could be owing to the presence of detrimental compounds like terpenoids, as stated earlier. We once reported that chloroform extract from the tar phase of tobacco extract decreased colonic cell proliferation, which in turn modulates the turnover of epithelium at the base of colonic crypt cells (Shin et al., 2003). This could at least partially explain the deteriorating effect on colitis development by the FCE1 extract.

Although we have suggested the potential use of nicotine or other protective alkaloids as found in the ethanol extract of tobacco smoke, it is also crucial to stress the general detrimental effects of smoking and the pathophysiological modulation of many dreadful diseases by various components in the smoke. There are $5 \times 10^9$ particles contained in 1 cc of mainstream smoke. In gas phase smoke, 99.9% of which is able to sieve through the cigarette filter, there are various gaseous compounds such as HCN, NO, and acetone (amounting to 0.5 mg/cigarette). In the cigarette we used in the present study, other than the 1.1 mg of nicotine, there are 15 mg of tar, and 12 mg equivalent of CO will be generated by the smoke. In addition, although tar and the total particulate matter (in the particulate phase) will be partially retained by the cigarette filter, the remaining amount to be inhaled or released to the environment still contains many detrimental compounds such as glycols, terpenoids, carboxylic acids, paraffin wax, and worst of all, the carcinogenic nitrosamines (amounting to 1.5–2 µg/cigarette) and aromatic hydrocarbons (amounting to 3.6 µg/cigarette, including 0.02 µg of benzo(a)pyrene). We had demonstrated in a chronic colitis-induced adenoma study that long-term treatment of 4% v/v tobacco smoke to DSS-treated mice increased angiogenesis and reduced the apoptosis/proliferation ratio, which resulted in a significantly increased incidence of inflammation-associated adenoma/adenocarcinoma formation (Liu et al., 2003). We further examined the
carcinogenic effects of the tobacco extract, and found that both ethanol and chloroform extracts from tobacco smoke would lead to elevation in the expression of the proto-oncogene c-myc in a gastric adenocarcinoma cell line, although such expression was found to be reduced by the same treatments in a well-differentiated colon adenocarcinoma cell line (Shin et al., 2003). To our surprise, we also discovered that nicotine, the “protective” component in the tobacco smoke component as shown in the present study, would lead to increase in proliferation in colon adenocarcinoma cells, with concurrent overexpression of the 5-lipoxygenase enzyme and an increase in epidermal growth factor receptor and c-Src phosphorylation levels, which eventually contributed to the promotion of tumor growth in nicotine-treated nude mice (Ye et al., 2004). These points together raise an important question about how to balance the “beneficial” and harmful effects of tobacco smoke components, including the seemingly “protective” nicotine. The implications being brought forth from findings of the present investigation actually target the discovery of potential novel anti-inflammatory agents and the underlying mechanisms that could be used in the treatment of colitis diseases.

In conclusion, tobacco smoke contains various active chemical components that exert differential effects in DNBS-induced colitis. When some of the damaging factors are removed from the smoke after it is passed through the filter, the net effect could be colonic protection. As a result, both filtered tobacco smoke and nicotine have been demonstrated to accelerate healing during active colitis, which also involves inhibition of neutrophil-derived free radicals and downregulation of the pro-inflammatory chemokine and cytokine protein synthesis in the colonic tissue. Nonetheless, it is remarkable that the vast number of detrimental substances present in tobacco smoke, including terpenoids, polyphenols, nitrosamines, and polycyclic hydrocarbons, plus the multiple systemic side effects of nicotine have to be considered in the search of an effective chemotherapeutic regimen for IBD. Perhaps the verification of the precise protective alkaloids contained in tobacco smoke could be of even a greater importance.

SUPPLEMENTARY DATA

Supplementary data are available online at www.toxsci.oxfordjournals.org.

REFERENCES


