

# Cigarette smoke exposure increases ulcerative colitis-associated colonic adenoma formation in mice

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**Both chronic ulcerative colitis and smoking are associated with colorectal cancer in humans. In the present study, we investigated the effects of cigarette smoke (CS) exposure on inflammation-associated tumorigenesis in the mouse colon. Male balb/c mice were allocated into six groups: control, CS (2%), CS (4%), colitis, colitis + CS (2%) and colitis + CS (4%). They were given water or 3% dextran sulfate sodium (DSS) in drinking water for 7 days to induce colitis, with or without 1 h daily exposure to 2 or 4% CS. They were then allowed to drink water for 14 days. The cycle of 7 day DSS ± CS/14 day H<sub>2</sub>O treatments were repeated twice. Mice were killed immediately or 1 month after the three cycles of treatments. Results indicated colonic adenoma was only found in the colitis group (one out of 11), Colitis + CS (2%) group (seven out of 12) and colitis + CS (4%) group (four out of five) 1 month after three cycles of DSS and/or CS treatment. CS exposure dose-dependently increased adenoma formation in mice with inflamed mucosa. CS exposure plus colitis was strongly associated with a high incidence of dysplasia ( $P < 0.01$ ) and adenocarcinoma formation ( $P < 0.01$ ) compared with induction of colitis alone. Colitis induced cell proliferation and apoptosis in colonic tissues. Cigarette smoking significantly attenuated the apoptotic effect by DSS probably via the induction of anti-apoptotic protein bcl-2. The ratio of apoptosis over proliferation was also significantly lower in the colitis + CS groups. Vascular endothelial growth factor and angiogenesis in the colon were also increased by cigarette smoking in animals with colitis. In conclusion, CS promotes inflammation-associated adenoma/adenocarcinoma formation in the mouse colon in a dose-dependent manner. This tumor development is associated with the inhibition of cellular apoptosis and supported by increased angiogenesis.**

## Introduction

Inflammation-associated neoplasia is one of the major pathways in the development of colorectal cancer. Epidemiological

studies have suggested that patients with chronic and extensive ulcerative colitis have a higher risk of developing colorectal cancer (1–3). The studies have associated the longevity of the inflammatory disease with an increase in the risk of cancer. Other factors are also involved in the process of colorectal carcinogenesis, including environmental factors such as cigarette smoke (CS). Previous studies have shown that smokers develop colorectal cancer more often than non-smokers (4–6), and that the number of smoking pack-years is closely related to adenomatous polyp formation. Chronic inflammation and cigarette smoking can therefore both separately promote colorectal carcinogenesis. However, cigarette smoking appears to provide some protection against the development of ulcerative colitis in humans, as most patients with ulcerative colitis are non-smokers (7). The paradoxical role of cigarette smoking in colonic inflammation and carcinogenesis and their possible interaction is worth study.

In this study, we determined whether CS exposure affected colonic inflammation, and to what extent its effects modified carcinogenesis in the colon, if at all. Dextran sulfate sodium (DSS) model was repeatedly administered to induce chronic colitis and perhaps colonic dysplasia and cancer in mice, which closely resembled ulcerative colitis disease and cancer in humans (8–10). Mice were also exposed to CS on a daily basis, to determine if cigarette smoking could affect the inflammation-associated neoplasia. The morphological changes including cell proliferation, apoptosis and angiogenesis in the colon at the pre- and post-cancer stages were examined to elucidate how, if at all, these changes affected the formation of cancer by cigarette smoking during ulcerative colitis in animals.

## Materials and methods

### *Animals and materials*

The study was approved by the Committee for Use of Live Animals for Teaching and Research of the University of Hong Kong. Male balb/c mice (6–8 weeks old) were fed a standard laboratory diet (Ralston Purina Co., Chicago, IL). They were kept in an air-conditioned room with controlled temperature ( $22 \pm 1^\circ\text{C}$ ), humidity (65–70%), and day/night cycle (12 h light, 12 h dark). All chemicals were purchased from Sigma Chemical (St Louis, MO) unless specified otherwise.

### *Induction of chronic ulcerative colitis and CS exposure*

The mice were divided into six groups: control, CS (2%), CS (4%), colitis, colitis + CS (2%) and colitis + CS (4%). Each group contained five to 12 animals. Chronic ulcerative colitis was induced by the repeated administration of DSS (ICN Pharmaceuticals, Costa Mesa, CA) in the colitis groups according to the method described by Okayasu *et al.* (8), with some modifications. The CS and the colitis + CS groups were simultaneously exposed to CS in a smoking chamber described previously (11). Briefly, commercial cigarettes (Camel, non-filter, R.J.Reynolds, Winston-Salem, NC) were used. During smoke exposure, the mice were put into a ventilated smoking chamber ( $39 \times 23.5 \times 21$  cm) with 2 or 4% CS (v/v, smoke/air). The smoke/air concentration in the chamber was kept constant by the use of peristaltic pumps (Masterflex, Cole Parmer Instrument, Niles, IL), which delivered

**Abbreviations:** CS, cigarette smoke; DSS, dextran sulfate sodium; PCNA, proliferating cell nuclear antigen; VEGF, vascular endothelial growth factor.

smoke and fresh air, respectively, at a different flow rate. Control mice were subjected to the same procedures, but breathed only fresh air. During the DSS treatment period, the groups were fed with water or 3% DSS in drinking water for 7 days, with 1 h daily exposure to 0 or 2 or 4% CS. The control group received H<sub>2</sub>O only; the two CS groups CS (2 or 4%) only; the colitis group DSS only; and the two colitis + CS groups DSS + CS (2 or 4%). They were then allowed to rest for 14 days before the next cycle began. The cycle of DSS/H<sub>2</sub>O ± CS treatments was repeated twice. Some of the mice were killed after three cycles, while the remainder was left for a further 1 month before death. The animals were killed by cervical dislocation and the colons were collected for assessment of inflammation and adenoma formation.

#### Assessment of colonic mucosa

A longitudinal section was obtained mainly from the lower half of the colon or around a polyp (which were found in the same part of the colon), if present, and fixed in a 10% formalin solution. After 24 h of fixation and paraffin embedding, 5 µm sections were cut and transferred onto glass slides. These sections were stained with hematoxylin–eosin to assess the morphology of the tissue. The severity of damage in the colonic tissue was graded according to the pathological score described by Okayasu *et al.* (8): viz. 0, normal; 1, focal inflammatory cell infiltration including polymorphonuclear leukocytes; 2, inflammatory cell infiltration, gland dropout and crypt abscess; and 3, mucosal ulceration.

#### Determination of the rate of cell proliferation and apoptosis in the colonic mucosa

The rate of cell proliferation was determined by staining for the proliferating cell nuclear antigen (PCNA) (12). Tissues were obtained from the lower half of the colon and were fixed, deparaffinized and hydrated. Following incubation with 0.3% H<sub>2</sub>O<sub>2</sub>–methanol, the sections were subjected to trypsin digestion for 30 min and then blocked by the addition of the normal serum in 0.05 M Tris–HCl buffer saline (TBS; pH 7.8) for 1 h. They were then incubated overnight with anti-PCNA mouse monoclonal antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C. On the following day a labeled streptavidin–biotin DAKO kit (DAKO, Glostrup, Denmark) and 3,3'-diaminobenzidine were used to visualize the PCNA-positive proliferating cells in the tissue. Mayer's hematoxylin was used to counterstain the sections. The positively stained mucosal cells were counted in six to eight randomized fields (400×) with the aid of a light microscope (Olympus, Melville, NY), and the average number of cells per 10 crypts was taken.

Apoptotic cells were visualized by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling method described previously (13). Briefly, the sections were deparaffinized and rehydrated. Proteinase K (Boehringer Mannheim, Mannheim, Germany) was added to digest the cells. TdT buffer solution (140 mM sodium cacodylate, 1 mg/ml BSA, 1 mM cobalt chloride in 30 mM Tris–HCl pH 7.0) containing 50 U/20 µl TdT (ICN Biochemicals) and 50 nmol/50 µl dUTP (Boehringer Mannheim) was added to the sections and incubated at 37°C for 90 min. The sections were then immersed in the buffer containing 300 mM sodium chloride and 30 mM sodium citrate. Apoptotic cells were labeled by the addition of peroxidase-conjugated streptavidin followed by 3,3'-diaminobenzidine. The apoptotic mucosal cells were counted in six to eight randomized fields (400×) with the aid of a light microscope, and the average was taken and expressed as the number of apoptotic cells per 10 crypts.

#### Assessment of angiogenesis in the colonic mucosa

The microvessels in the colonic mucosa were identified by immunohistochemical staining with the von Willebrand factor antibody (DAKO) (14). The staining procedure was the same as the PCNA staining described above, except the primary antibody was changed to the von Willebrand factor (1:200). The endothelial cells in the blood vessels were labeled by the addition of peroxidase-conjugated streptavidin followed by 3,3'-diaminobenzidine. The number of blood vessels was counted in six randomized fields (200×) in the colonic mucosa using a light microscope, and the amount was expressed as number per mm<sup>2</sup>.

#### Detection of bcl-2 and vascular endothelial growth factor (VEGF) protein expression by western blotting

Expression of bcl-2, an anti-apoptotic protein, and VEGF, a mediator of angiogenesis, were determined in the colon of mice 1 month after three DSS cycles. The colonic tissues obtained from the lower half of the colon were homogenized for 30 s in RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM sodium chloride, 0.5% α-cholate, 0.1% SDS, 2 mM EDTA, 1% Triton X-100 and 10% glycerol) containing 1.0 mM phenylmethylsulfonyl fluoride and 1 µg/ml aprotinin. The samples were then centrifuged at 17 968 g for 20 min at 4°C and the supernatant containing 70 µg of the protein was denatured and separated by electrophoresis on a sodium dodecyl polyacrylamide gel. The protein was then transferred to a nitrocellulose membrane (Bio-Rad

Hercules, CA) probed either with rabbit polyclonal antibody against bcl-2 protein (1:300; Santa Cruz) or mouse monoclonal antibody against VEGF protein (1:500; Santa Cruz). Membranes were then developed by the ECL chemiluminescence and exposed on an X-ray film. Quantification of the bands on the film was carried out by video densitometry (Gel Doc 1000, Bio-Rad).

#### Statistical analysis

Results were expressed as mean ± SEM. Means were compared by the use of ANOVA, followed by the Student's *t*-test or the  $\chi^2$  test. A *P* value < 0.05 was considered to be statistically significant.

## Results

### Morphology of the colonic tissue

Chronic DSS treatment produced colonic inflammation in mice, which was characterized by edema, shortening of the colon and inflammatory cell infiltration (Table I). Addition of CS exposure did not affect the inflammation induced by DSS. No dysplasia/adenoma was found in the colonic tissue after three cycles of DSS treatment. However, 1 month after the three DSS cycles, dysplasia and carcinoma were found in the colitis and colitis + CS (2 or 4%) groups (Figure 1B and C). Adenomatous polyps were also demonstrated in the colon mucosa (Figure 1D). These were found entirely in the lower half of the colon. Table II shows the number of mice with colonic dysplasia and/or adenomatous polyps. CS (2 or 4%) increased the incidence of adenomatous polyps dose-dependently from 9 to 58 and 80% (*P* < 0.01), respectively, in mice treated by DSS. Colonic dysplasia was more frequent in the colitis + CS (2 or 4%) groups than in the colitis group (*P* < 0.01), and was a phenomenon found entirely in the colon with polyps. Among the polyps produced in the colitis + CS (2%) group that was associated with dysplasia (five out of seven), three of them are adenocarcinomas, as revealed by hematoxylin–eosin staining.

### Cell proliferation and apoptosis

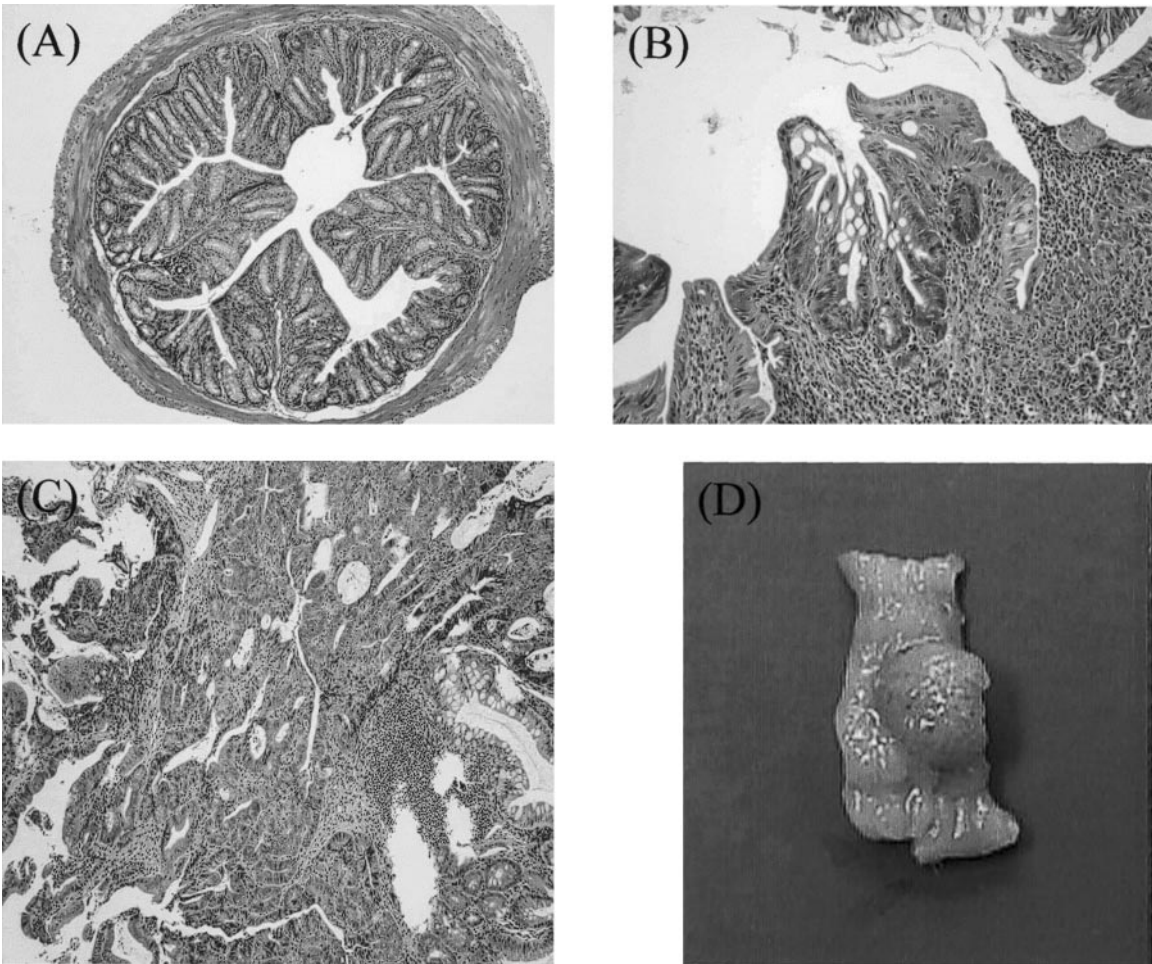
The number of proliferating cells found in the section obtained from the lower half of the colon is shown in Figure 2. Proliferation was induced in both the colitis and colitis + CS (2%) group throughout the whole tissue section after three DSS cycles of treatment (*P* < 0.05 when compared with the control group). Cellular proliferation was more evidently induced 1 month after the colitis induction, in which not only the colitis or colitis + CS (2 and 4%) group produced more colonic proliferating cells, but also the CS groups induced dose-dependently more proliferating cells in the colonic mucosa when compared with the corresponding control group.

**Table I.** Colon assessment in mice with chronic colitis with or without CS exposure after three cycles of DSS treatment

	Control group ( <i>n</i> = 7)	Colitis group ( <i>n</i> = 7)	Colitis + CS (2%) group ( <i>n</i> = 7)
CW/BW (mg/mg)	7.78 ± 0.82	8.69 ± 0.39	9.48 ± 0.67
CL/BW (cm/mg)	0.33 ± 0.02	0.23 ± 0.02 <sup>a</sup>	0.24 ± 0.01 <sup>a</sup>
Pathological score	0.00 ± 0.00	1.75 ± 0.25 <sup>a</sup>	1.50 ± 0.29 <sup>a</sup>

CW/BW, colon weight per body weight, indicating edema; CL/BW, colon length per body weight. Pathological score represents the severity of inflammation in the colon.

<sup>a</sup>*P* < 0.05 when compared with the control group.



**Fig. 1.** Histological demonstration of colon with (A) normal mucosa (original magnification 100×), (B) mild dysplasia of the cryptal epithelial cells in the mucosa (original magnification 200×) and (C) adenocarcinoma at the center of the mucosa (original magnification 200×); (D) macroscopic appearance of an adenoma in the colon of the colitis + CS (2%) group (size: 0.5 cm in diameter).

**Table II.** Incidence of dysplasia/adenomatous polyps in mice with chronic colitis with or without CS exposure 1 month after three cycles of DSS treatment

	Colitis group (n = 11)	Colitis + CS (2%) group (n = 12)	Colitis + CS (4%) group (n = 5)
Dysplasia	1 (9.1%)	5 (45.5%) <sup>a</sup>	4 (80%) <sup>a</sup>
Adenomatous polyps	1 (9.1%)	7 (58.3%) <sup>a</sup>	4 (80%) <sup>a</sup>

No dysplasia or carcinoma was found in the control (see Figure 1A) or CS groups.

<sup>a</sup>*P* < 0.01 when compared with the colitis group.

Colitis markedly increased apoptotic colonic cells mainly in the epithelium of the colonic mucosa (Figure 3). However, fewer apoptotic cells were found in the colon 1 month later. The addition of CS exposure (2%) reduced the induction of apoptotic cells by DSS immediately after three cycles of DSS, and apoptosis was significantly inhibited 1 month after treatment in both colitis + CS (2%) and colitis + CS (4%) groups when compared with the colitis group (*P* < 0.05). The ratio of apoptosis to proliferation is shown in Figure 4. There were no significant differences between the different groups immediately after three DSS cycles. However, 1 month later, the ratio

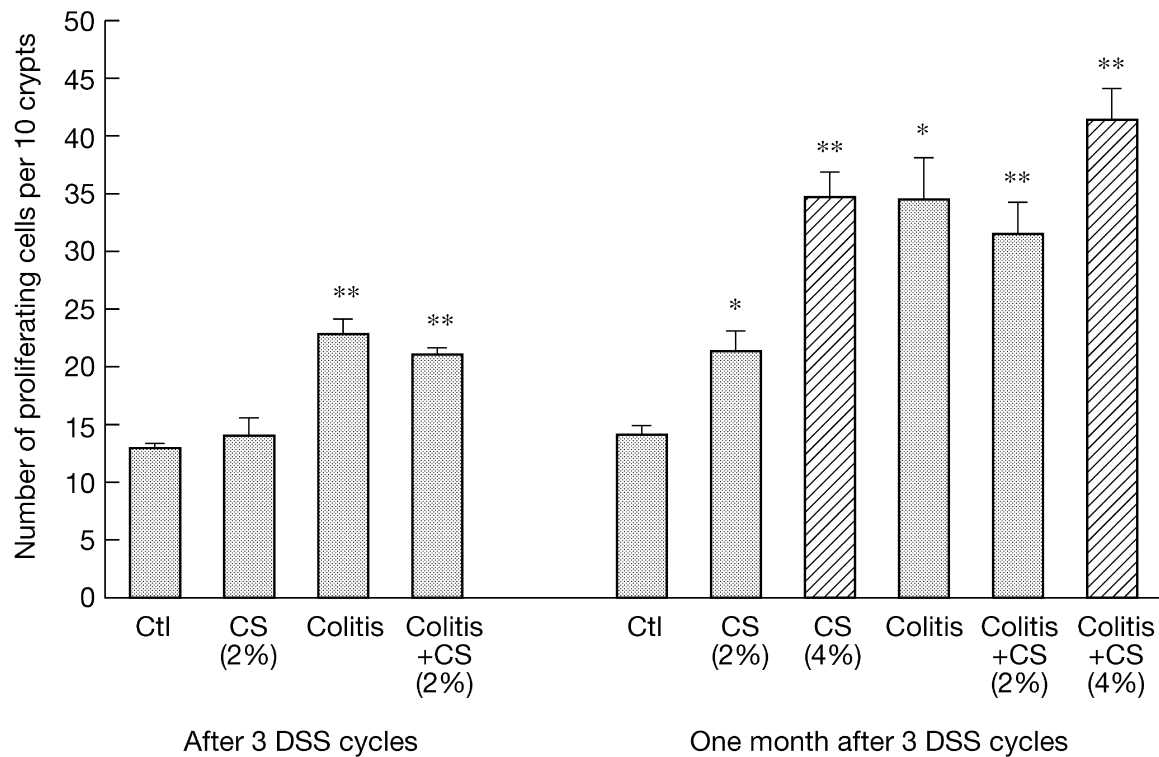
decreased in the groups exposed to CS (4%) or colitis induction (*P* < 0.01 or *P* < 0.05 versus control), with a further reduction of the ratio in the colitis + CS (2 or 4%) groups (*P* < 0.01 versus control; *P* < 0.05 versus colitis group).

*Angiogenesis in the colonic tissue*

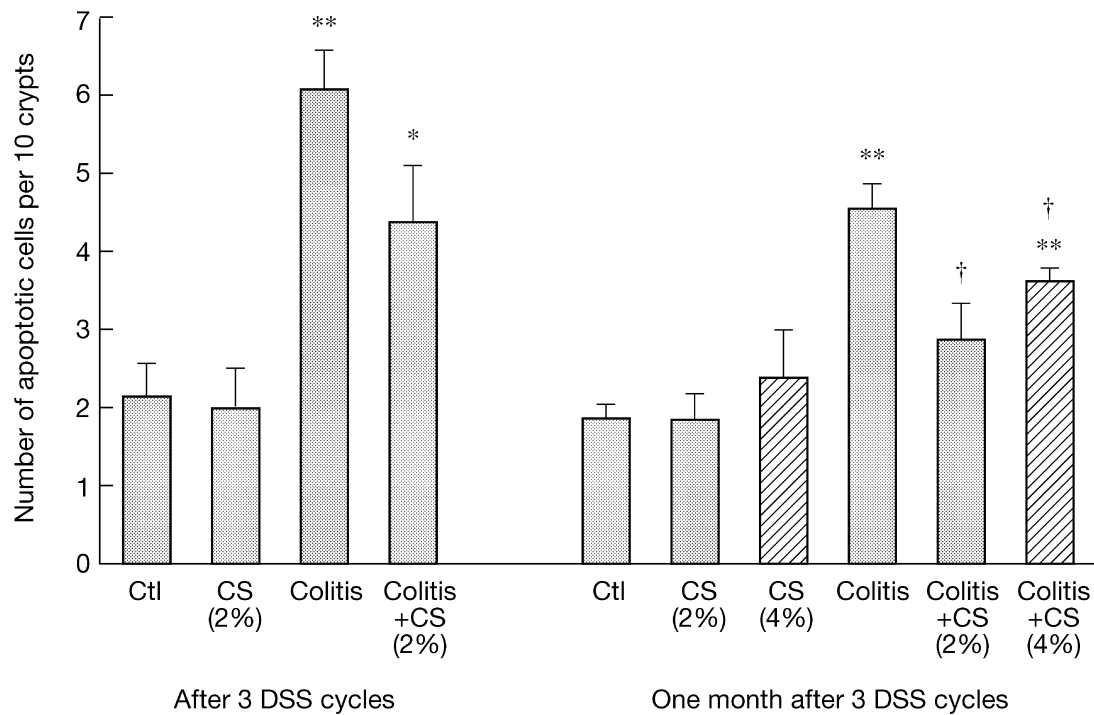
The number of blood vessels in the colonic mucosa in each treatment group is shown in Figure 5. Colitis induction induced a significantly higher number of blood vessels in mice (*P* < 0.05), with further induction when combined with CS exposure (*P* < 0.01 versus control). Moreover, 1 month after the DSS cycles, the significant increase sustained in the mice with colitis and colitis plus CS exposure (2 or 4%) (*P* < 0.05 and *P* < 0.01 respectively, versus control). CS exposure dose-dependently induced further angiogenesis in animals with colitis, with a significant induction of angiogenesis in the colitis + CS (4%) group when compared with the colitis group (*P* < 0.05).

*Bcl-2 and VEGF expression in the colonic tissue*

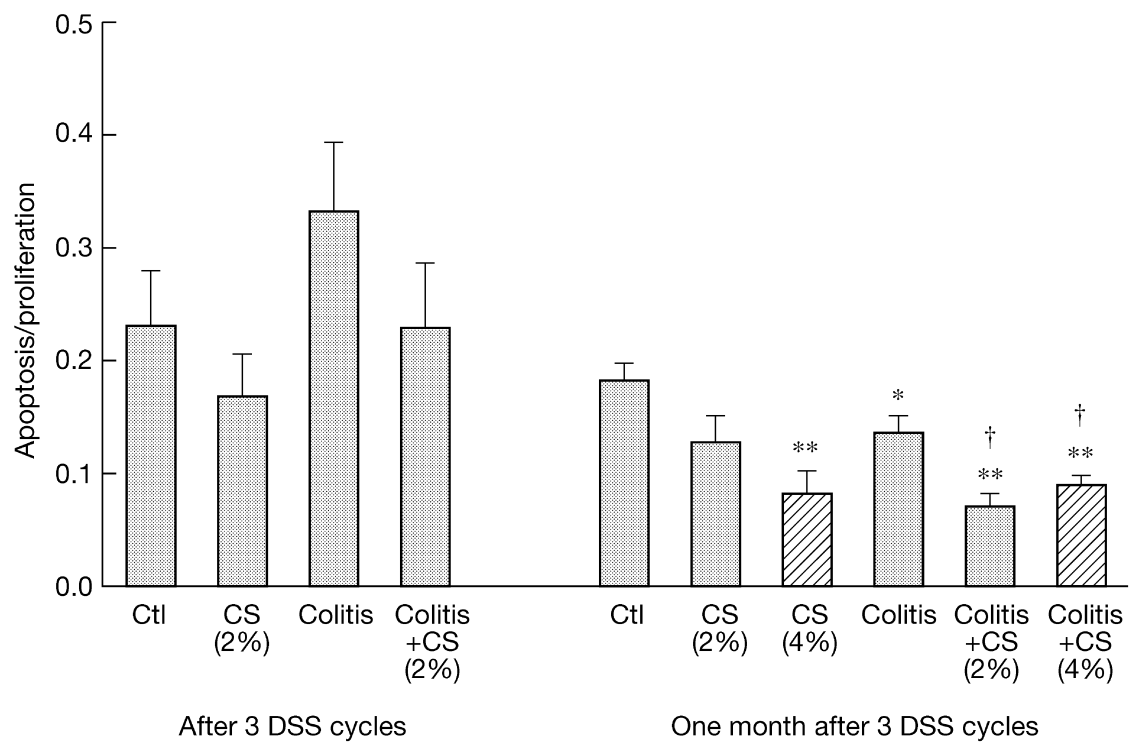
Colonic bcl-2 expression was significantly induced in the colitis + CS group 1 month after DSS treatments (*P* < 0.05 versus control, Figure 6A). VEGF protein expression was also increased in the colitis + CS group (*P* < 0.05 versus control, Figure 6B).



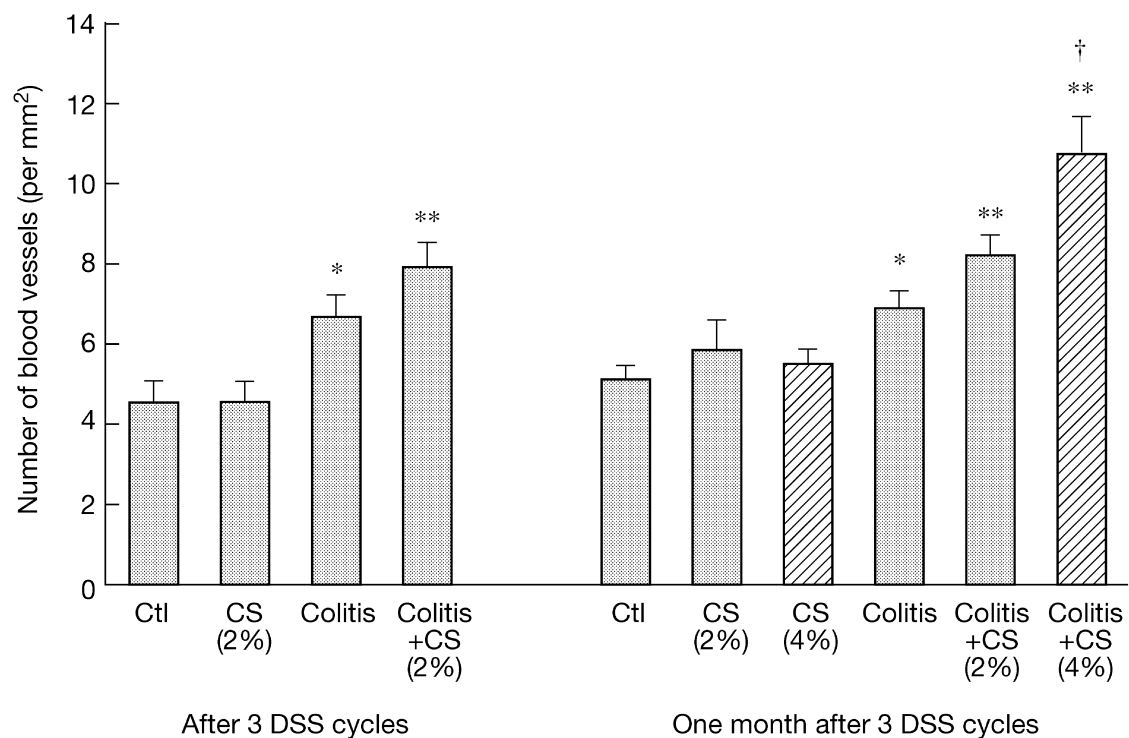
**Fig. 2.** Number of proliferating cells in colonic mucosa from mice killed immediately or 1 month after three cycles of DSS treatment. Ctl group, control group, water treatment only; CS (2 or 4%) groups, CS exposure (2 or 4%, smoke/air, v/v); colitis group, DSS treatment only; colitis + CS (2 or 4%) groups, DSS and CS exposure (2 or 4%, smoke/air, v/v) ( $n = 7-12$  mice/group). Data were expressed as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$  when compared with the corresponding control group.



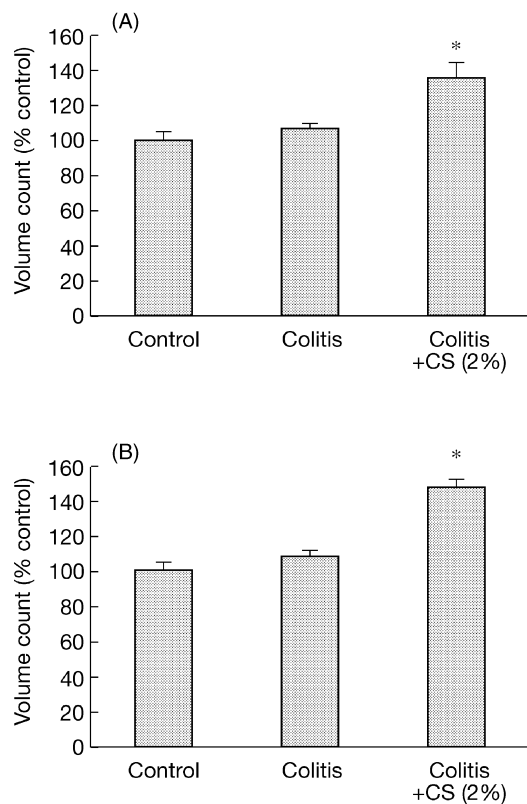
**Fig. 3.** Number of apoptotic cells in colonic mucosa from mice killed immediately or 1 month after three cycles of DSS treatment. Ctl group, control group, water treatment only; CS (2 or 4%) groups, CS exposure (2 or 4%, smoke/air, v/v); colitis group, DSS treatment only; colitis + CS (2 or 4%) groups, DSS and CS exposure (2 or 4%, smoke/air, v/v) ( $n = 7-12$  mice/group). Data were expressed as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$  when compared with the corresponding control group. † $P < 0.05$  when compared with the corresponding colitis group.



**Fig. 4.** Ratio of apoptosis over proliferation in colonic mucosa from mice killed immediately or 1 month after three cycles of DSS treatment. Ctl group, control group, water treatment only; CS (2 or 4%) groups, CS exposure (2 or 4%, smoke/air, v/v); colitis group, DSS treatment only; colitis + CS (2 or 4%) groups, DSS and CS exposure (2 or 4%, smoke/air, v/v) ( $n = 7-12$  mice/group). Data were expressed as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$  when compared with the corresponding control group. † $P < 0.05$  when compared with the corresponding colitis group.



**Fig. 5.** Number of blood vessels in colonic mucosa from mice killed immediately or 1 month after three cycles of DSS treatment. Ctl group, control group, water treatment only; CS (2 or 4%) groups, CS exposure (2 or 4%, smoke/air, v/v); colitis group, DSS treatment only; colitis + CS (2 or 4%) groups, DSS and CS exposure (2 or 4%, smoke/air, v/v) ( $n = 7-12$  mice/group). Data were expressed as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$  when compared with the corresponding control group. † $P < 0.05$  when compared with the corresponding colitis group.



**Fig. 6.** Protein expression of (A) bcl-2 and (B) VEGF in the colonic tissue from mice 1 month after three cycles of DSS treatment. Control group, water treatment only; colitis group, DSS treatment only; colitis + CS (2%) groups, DSS and CS exposure (2%, smoke/air, v/v) ( $n = 7-12$  mice/group). Data were expressed as mean  $\pm$  SEM. \* $P < 0.05$  when compared with the corresponding control group.

## Discussion

This is the first report demonstrating the effect of CS in promoting inflammation-associated carcinogenesis in the colon. Epidemiological studies have shown that cigarette smoking is a risk factor for carcinogenesis in various organs. Besides its fully documented carcinogenic effect on the respiratory system (15,16), cigarette smoking is also significantly associated with tumorigenesis in the gastrointestinal system, including the oral cavity, esophagus, stomach, ileum and colon (17–21). On the other hand, gastrointestinal tumorigenesis is strongly associated with chronic inflammation (22,23). Chronic inflammation is characterized by a repeated process of inflammatory injury and tissue repair, in which the turnover of cells is rapid and massive. If there is an error in the repair of DNA damage, the chance of generating a cell with mutant DNA is greater, thus initiating cancer development. In the current study, chronic exposure to CS did not aggravate or prevent the colonic inflammation induced by DSS treatments, in terms of edema or inflammatory cell infiltration, nor did it produce adenoma by itself. Our preliminary study showed that when mice were exposed to one DSS cycle, CS exposure did not aggravate or prevent colonic inflammation but it reduced lipid peroxidation in the colon. The colonic mucosa displayed similar microscopic phenomena such as edema and inflammatory cell infiltration in the colitis and the colitis + CS groups (unpublished findings). In short, at the present dosage and

duration, exposure to CS failed to provide the mice with any significant protection against DSS-induced ulcerative colitis. Although it is not in line with clinical findings (7,24), the inflammation in the current animal model occurred in a short period of time, and the inflammatory responses were also much more severe. Under these acute and severe experimental conditions, if the protective effect of CS is mild as shown in human cases, any significant protective effect may have been hindered and unobserved in the current study. However, CS exposure promoted carcinogenesis in mice with chronic ulcerative colitis in a dose-dependent manner, indicating that the promotion of tumor formation in the colon by CS exposure was probably not due to an increase in the severity of the inflammation. Instead, it is likely that exposure to CS provided an abundant source of genotoxic carcinogens (25). DNA repair errors are more likely to occur during the rapid turnover of cells in the inflamed colonic mucosa. As a result, a significant number of mutant cells could have been generated for the development of dysplasia and adenoma formation in the colitis + CS groups. In addition, exposure to a higher concentration of CS (4%) would provide more chance for replication error in mucosal regeneration, which led to a higher incidence of adenoma formation in the colitis + CS (4%) group. Moreover, only one mouse (out of 12) with adenoma was found in the colitis group, indicating that inflammation alone is a weak inducer of carcinogenesis in the colon. Additional factors are needed for the full-blown development of cancer.

Morphologically, dysplasia and adenomas occur at an early stage in colorectal carcinogenesis (26). Previous studies indicated that the administration of DSS alone, either at a higher dosage (5%) or for a longer period (180 days), generated dysplasia and/or adenocarcinoma in ~30% of the mice concerned (9,10). In the present study, a lower dosage of DSS was administered, and dysplasia was detected in <10% of the mice. However, the addition of CS significantly increased the incidence of dysplasia found in the colon ( $P < 0.01$  compared with the colitis groups), and was strongly associated with the development of adenocarcinoma. This may reflect more severe pathological changes in mice with simultaneous DSS and CS treatments.

Moreover, in the present study, the prevalence of adenoma and dysplasia/adenocarcinoma was enhanced in the colitis + CS groups 1 month after the three cycles of DSS treatment. This indicates that the 1 month delay was important for tumor growth. Indeed, the number of apoptotic cells decreased significantly while the proliferation rate increased, leading to a lower ratio of apoptosis/proliferation 1 month after DSS treatments. This effect was more obvious in the colitis + CS groups, which developed more adenomas. Our data indicated that although DSS treatment initiated tissue damage, and thereby cell proliferation and apoptosis, the addition of CS exposure upregulated the expression of anti-apoptotic protein bcl-2 in the DSS + CS group, which could contribute to a reduced level of apoptosis in these animals. The reduction of cell loss, together with the induction of proliferation, would favor the development of adenoma in the colon of animals exposed to smoke. A further induction of cellular proliferation and an inhibition of apoptosis by 4% CS could also contribute to more tumor development in the DSS + CS (4%) group. In humans, the level of apoptosis has been related to the invasiveness and metastatic activity in colorectal cancer (27). Lower levels of apoptosis are closely associated with tumor growth. This could be achieved by persistent induction of bcl-2

expression in cancer cells (28,29). The current findings of the association between downregulation of apoptosis and bcl-2 induction in the colitis + CS groups are relevant to those in human cases. In addition, angiogenesis was induced in the inflamed colon tissue. Angiogenesis is important for wound healing, but it also plays a major role in tumor growth (30,31). VEGF is the protein responsible for the enhancement of angiogenesis, tumor growth and metastasis, and is widely expressed in different cancers along the gastrointestinal tract (32,33). The induction of angiogenesis by colitis and further by smoking, with the associated induction of VEGF protein, was evident only in the colitis + CS groups 1 month after DSS treatments. These findings indicate that smoke exposure favored the formation of new blood vessels, which would support further tumor growth and cancer development in these animals.

To conclude, neither cigarette smoking nor mild inflammation in isolation has significant effect on tumorigenesis in the colon. However, a combination of both even in a relatively short term can markedly provoke pathologic change, which is probably caused by the inhibition of apoptosis and upregulation of angiogenesis in the colon.

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