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Cyclooxygenase-2 in cancer cells and macrophages induces colon cancer cell growth by cigarette smoke extract

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Abstract

Cigarette smoking, cyclooxygenase-2 (COX-2) and macrophages are independently associated with colorectal cancer. In the present study, cigarette smoke ethanol extract was applied to colon cancer cells (SW1116) or indirectly via activated macrophages (THP-1 cells) to attest their effects on cancer cell proliferation and tumor growth both in vitro and in vivo. Ethanol extract induced COX-2 expression in SW1116 and THP-1 cells. Combination of THP-1 pre-incubated medium and ethanol extract further potentiated COX-2 expression and proliferation of SW1116 cells.

Tumor growth in nude mice was positively associated with the medium and/or ethanol extract treatments, together with the up-regulation of cell proliferation and angiogenesis, and down-regulation of apoptosis. Application of a COX-2 inhibitor (SC236) reduced tumor growth as well as cell proliferation and angiogenesis. These actions are partially depended on the decrease of COX-2 expression. Taken together, inhibition of COX-2 activity may have significant implication to prevent colon cancer in smokers.

Keywords: Cyclooxygenase-2; Cigarette smoke; Colon cancer

1. Introduction

Epidemiological studies show that smokers have a high risk of colonic adenoma and hyperplastic polyp (Martinez et al., 1997; Potter et al., 1999) and also colorectal carcinoma (Limburg et al., 2003; Newcomb et al., 1995; Slattery et al., 2003; Terry et al., 2001). Our previous study also indicates that cigarette smoke exposure can promote colitis-associated neoplasia (Liu et al., 2003). As cigarette smoke does not have a direct access to the colon, it would however act through the blood stream to transfer carcinogens or carcinogenic stimuli to colonic epithelial cells and activate pathological alternations. On the other hand, cyclooxygenase-2 (COX-2) is highly expressed in colon cancer in humans, and the expression appears early in the process of carcinogenesis (Eberhart et al., 1994; Hao et al., 1999; Sano et al., 1995). As a source of COX-2, macrophage, when activated, is a good candidate for activating the growth of epidermal cells, especially during inflammatory processes. It is likely that cigarette smoke could directly stimulate tumor growth through the expression of COX-2 in cancer cells or indirectly activate via macrophages during pathological changes in the colon.

Indeed, previous study shows that the majority of malignant tumors contain numerous macrophages, which are defined as tumor-associated macrophages (Ohno et al., 2003). Pro-tumor proteins such as vascular endothelial growth factor (VEGF), colony stimulating factor-1 and matrix metalloproteinases can be produced from these intracellular molecules (Chambers et al., 1997; Davies et

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al., 1993; Eberhart et al., 1994). Increase in expression of COX-2 by tumor-associated macrophages is also observed in both ovarian and colon cancers (Bamba et al., 1999; Klimp et al., 2001). These growth factors and cytokines are thought to promote cellular proliferation and angiogenesis in tumors. However, the tumor-associated macrophages are not characterized phenotypically and how they contribute to tumor development especially by cigarette smoke remain unclear.

The present study is aimed at demonstrating the direct or indirect tumorigenic effect of cigarette smoke extract on colon cancer cells. In this regard, cigarette smoke ethanol extract was either directly applied to the colon adenocarcinoma SW1116 cells or indirectly incubated with activated THP-1 cells (macrophages), to study whether cigarette smoke could affect colon cancer growth through activation of COX-2 expression in cancer cells or released from macrophages.

2. Materials and methods

2.1. Cell cultures

THP-1 (TIB-202), a human monocyte cell line was purchased from American Type Culture Collection (ATCC, Manassas, Virginia, USA). Cells were grown in a RPMI 1640 medium with 10% fetal bovine serum. They were allowed to differentiate into macrophages by addition of phorbol 12-myristate 13-acetate (100 nM) for 24 h before study. SW1116 (CCL-233), a human colon adenocarcinoma cell line was also obtained from ATCC. Cells were grown in a RPMI 1640 medium with 10% fetal bovine serum.

2.2. Cigarette smoke extract preparation

Cigarette smoke extract was prepared and administered to cultured cells. Camel non-filter cigarettes (R.J. Reynolds, Winston-Salem, NC, USA) were used in the extraction, as mentioned previously (Ma et al., 2000; Shin et al., 2002). Smoke generated from a burning cigarette was pumped into four flasks of 95% ethanol successively. The ethanol solvent, containing the cigarette smoke dissolvent, was then evaporated, and the residue was further dissolved in absolute chloroform. The insoluble part from the chloroform was therefore referred as the ethanol extract of cigarette smoke. Dimethyl sulfoxide was used to dissolve the ethanol extract at a concentration of 100 mg/ml, before the extract was prepared for cell incubation.

The components in the ethanol extract of cigarette smoke had been studied by gas chromatography and mass spectrometry previously in our laboratory (Chow et al., 1997). Alkaloids, such as nicotine, were found to be the major component in the extract. This extract was subsequently used in the cell culture experiment to examine the direct and indirect effects of cigarette smoke on colon cancer cell growth.

2.3. Treatment of smoke extract and macrophage pre-incubated medium

In the first part of the study, SW1116 cells were incubated directly with the ethanol extract of cigarette smoke (10 or 100 μg/ml) for 18 h. Dimethyl sulfoxide (0.1%) was used as a control. After incubation, cells were collected for the determination of cellular proliferation, COX-2 expression and also xenograft implantation into nude mice. To elucidate the possibility of an indirect effect of cigarette smoke on colon cancer cell growth via macrophages, ethanol extract (10 or 100 μg/ml) was added to the activated THP-1 macrophages and incubated for 5 h for assessment of cellular proliferation and COX-2 expression. In another cell subpopulation, the culture medium was taken out from the ethanol extract-pretreated macrophages after 5 h of incubation and then transferred to the SW1116 cells for 18 h. After the incubation, similar parameters were measured, as previously described.

2.4. [^{3}H]Thymidine incorporation for cell proliferation assay in cultured cells

The proliferating effect of cigarette smoke extract on colon cancer cells and macrophages was determined by the [^{3}H]thymidine incorporation assay (Tones et al., 1988). Cells were seeded in a 24-well plate at a concentration of 1.2×10^5 per well. Ethanol extract was added to the wells containing SW1116 cells or activated THP-1 cells and incubated for 18 and 5 h, respectively at 37 °C. Dimethyl sulfoxide (0.1%) was used as a control. The extract was removed after incubation and then 1 ml of [^{3}H]thymidine solution (0.5 μCi per well) was added for 5 h at 37 °C. The incubation solution was then aspirated and washed with ice-cold 0.15 M sodium chloride. Cells were then incubated with 0.5 ml 10% trichloroacetic acid for 15 min at room temperature. After washing with distilled water, sodium dodecyl sulfate (0.5 ml, 1%) was then added to wells and incubated for 15 min at 37 °C. Cell lysates were transferred to scintillation vials, followed by addition of 9 ml scintillation fluid. The amount of [^{3}H]thymidine incorporated was measured using a liquid scintillation spectrometry on a beta-counter (LS6500, Beckman Instruments, Fullerton, California, USA).

2.5. Colon cancer xenograft implantation

SW1116 cells were collected following 18-h incubation with ethanol extract, or with THP-1 pre-cultured medium with or without ethanol extract, or with dimethyl sulfoxide (0.1%) as a control. Viable cells were resuspended in the medium and a volume of 0.2 ml of cells (2×10^5) was collected in aliquot. Simultaneously the balb/c nu/nu (nude) mice were anesthetized by the intraperitoneal injection of xylazine (10 mg/ml)/2% ketamine mixture at a dose of 70 mg/kg. Cells were then injected into the right flank of nude mice. The width and length of the tumor in millimeters were measured by a caliper once in 3–4 days starting from the tenth day after the implantation, such that the volume could be calculated according to the following formula: $Volume = d^2 \times D/2$ mm^3, where d was the shorter width and D was the longer length (Pratesi et al., 2002). The mice were killed by the end of 33 days, and the tumor implant from each mouse was taken out for protein assay and histology assessment after fixation in formalin.

2.6. Immunohistochemistry for determination of cellular proliferation, apoptosis and angiogenesis in tumors

The procedure for the determination of cell proliferation was described previously (Ezaki, 2000). Briefly, tissue sections were heated in citrate buffer (0.01 M, pH 6.0) at 80 °C for 15 min. After heating, sections were subjected to pepsin (0.005%)/HCl (0.01 N, pH 2.0) digestion. After blocking with normal serum, the monoclonal anti-proliferating cell nuclear antigen (PCNA) anti-

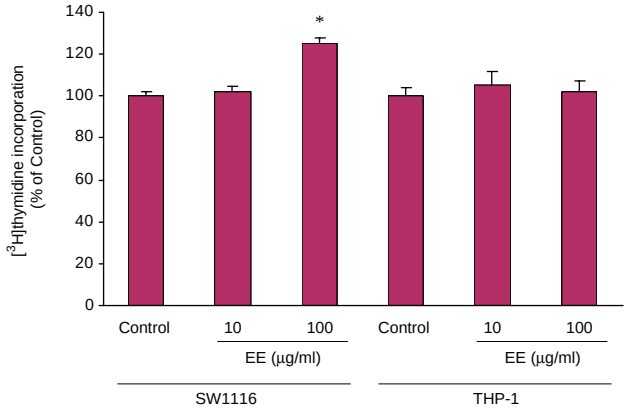

Fig. 1. [^{3}H]Thymidine incorporation by SW1116 cells or THP-1 cells after incubation with cigarette smoke extract (EE) for 18 h or 5 h respectively. Values were expressed as mean ± S.E.M. *P < 0.05 when compared with the corresponding control group.

body (1 : 200) was applied to sections for 2 h at room temperature. The antibody-labeled cells were visualized by using the labeled streptavidin-biotin DAKO kit (DAKO, Glostrup, Denmark), in combination with 3,3′-diaminobenzidine (DAB, 0.4 mg/ml). The number of proliferating cells was recorded in 6–8 randomized fields (× 400) under the microscope and it was expressed as the number per 2 fields.

Apoptotic cells were visualized by the terminal deoxy-transferase (TdT)-mediated dUTP biotin nick end labeling (TUNEL) method, as mentioned before (Gavrieli et al., 1992). Briefly, tissue sections were subjected to digestion by proteinase K. TdT buffer solution, which was 30 mM Tris–HCl (pH 7.0) with 140 mM sodium cacodylate, 1 mg/ml bovine serum albumin, 1 mM cobalt chloride, was added together with 50 U TdT and 5 nM dUTP to tissue sections and incubated at 37 °C for 90 min. After that the reaction was terminated by 30 mM sodium citrate buffer. Peroxidase-conjugated streptavidin from the DAKO kit was added and followed by the addition of DAB. The number of apoptotic cells was counted under a microscope (× 400) and was expressed as the number per 2 fields.

Microvessels in the colonic mucosa were identified by staining with the von Willebrand factor antibody (Augustin et al., 1995). Tissue sections were incubated with 0.3% H_2O_2–methanol for 15 min, followed by trypsin (1 mg/ml) digestion for 30 min. After blocking with normal serum, sections were then incubated overnight with the von Willebrand factor antibody (1 : 200). On the following day, the DAKO kit in combination with DAB was used to visualize endothelial cells of the blood vessels. The number of blood vessels was counted in 6 randomized fields (× 200) in the colonic tissue using a light microscope, and the amount was expressed as number per mm^2.

2.7. Determination of COX-2, bcl-2 and VEGF by Western blot

Tumor tissues from mice were homogenized for 30 s in a RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM sodium chloride, 0.5% α-cholate, 0.1% sodium dodecyl sulfate, 2 mM EDTA, 1% Triton X-100 and 10% glycerol) containing 1.0 mM phenylmethylsulfonyl fluoride and 1 μg/ml aprotinin. The homogenate was then centrifuged at 17 698 × g for 20 min at 4 °C and the protein content of the supernatant was determined by the Dye-reagent protein assay, as described previously (Shin et al., 2002). Similar procedures were performed on the cell lysates of SW1116 or THP-1 cells. Samples containing 70 μg of the protein was denatured and separated by electrophoresis on a sodium dodecyl polyacrylamide gel. The separated protein was then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, California, USA) probed with different diluted primary antibodies (Santa Cruz Biotechnology, Santa Cruz, California, USA): goat polyclonal anti-COX-2 (1 : 1000); mouse monoclonal anti-bcl-2 (1 : 350); mouse monoclonal anti-VEGF (1 : 300). On the next day, after incubation with secondary antibodies, protein bands on the membranes were then developed by chemiluminescence detection system and exposed on an X-ray film. Quantification of bands on the film was carried out by video densitometry and the data were finally expressed as % of the control or vehicle treatments.

2.8. Inhibition of COX-2 by SC236

A highly specific COX-2 inhibitor, SC236 (4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzenesulfonamide) (Pharmacia, Peapack, New Jersey, USA), was dissolved in absolute ethanol and diluted with normal saline (1 : 99) containing 5% Tween 80. The drug was then injected into nude mice at a dose of 2.0 mg/kg three times a week after the tumor implantation. The vehicle for treatment was the dissolvent for SC236 but without the drug itself.

2.9. Statistical analysis

The statistical methods applied in the study were described previously (Armitage and Berry, 1987). Data were expressed as means ± S.E.M. (standard error of mean). The difference between treatment groups was analyzed using the two-way statistical analysis of variance (ANOVA), followed by unpaired Student's t test. P-values less than 0.05 were considered significant.

3. Results

3.1. Effect of cigarette smoke extract on cellular proliferation

Fig. 1 shows the [^{3}H]thymidine incorporation by adenocarcinoma SW1116 or macrophages THP-1 cells after incubation with

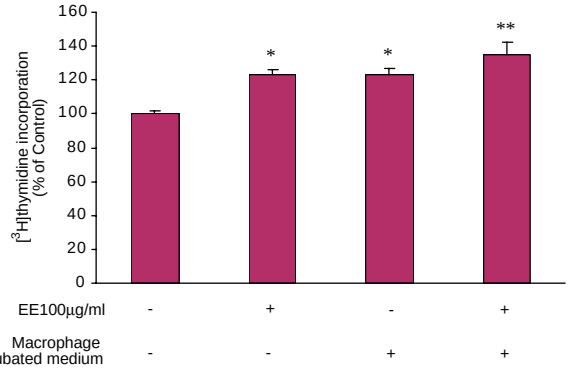

Fig. 2. [^{3}H]Thymidine incorporation by SW1116 cells after 18 h incubation with cigarette smoke extract (EE) and/or macrophage pre-incubated medium. The medium was derived from macrophages (activated THP-1 cells) after incubation with or without EE for 5 h before incubation with SW1116 cells. Values were expressed as mean ± S.E.M. *P < 0.05, **P < 0.01 when compared with the control group.

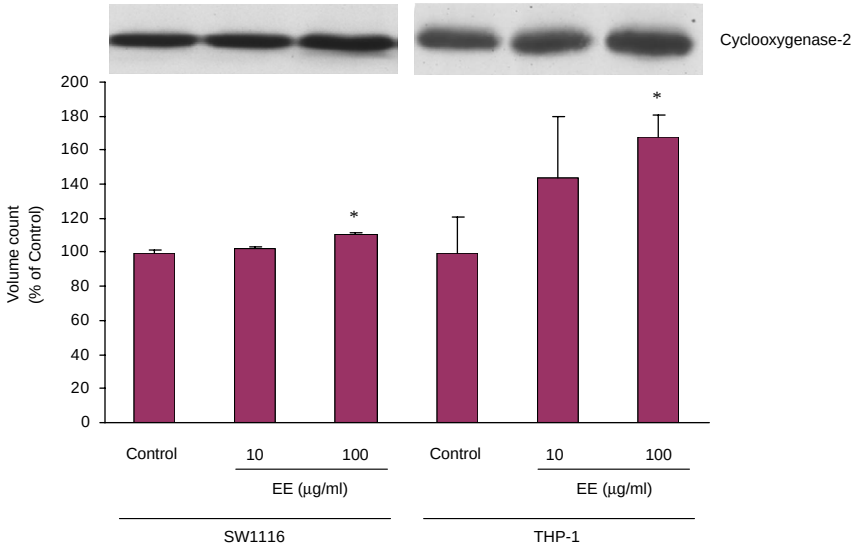

Fig. 3. Cyclooxygenase-2 expression by SW1116 or THP-1 cells after incubation with cigarette smoke ethanol extract (EE) for 18 and 5 h respectively. Values were expressed as mean ± S.E.M. *$P<0.05$ when compared with the corresponding control group.

ethanol extract. The highest dose of ethanol extract significantly induced the proliferation of SW1116 cells by around 30% as compared with the control group ($P<0.05$). However, in another experiment the extract at either 10 or 100 μg/ml failed to up-regulate proliferation of THP-1 cells, when compared with the corresponding control group.

To illustrate the interaction between smoke extract and macrophages on colon cancer cell growth, a separate experiment was performed. Fig. 2 shows the [^{3}H]thymidine incorporation by SW1116 cells after treatment with ethanol extract (100 μg/ml) or the medium pre-incubated with macrophages (with or without ethanol extract). Result indicated that either ethanol extract or macrophage pre-incubated medium alone had a significant induction of cellular proliferation of SW1116 cells, when compared with the control without any treatment ($P<0.05$). The macrophage pre-incubated medium containing ethanol extract (pre-incubated for 5 h) further potentiated cellular proliferation of SW1116 cells after incubation of 18 h ($P<0.01$) when compared with the control group.

3.2. Effect of cigarette smoke extract on COX-2 expression

Fig. 3 shows the expression of COX-2 by SW1116 or THP-1 cells after incubation with the ethanol extract. The higher dose of ethanol extract (100 μg/ml) significantly increased the expression of COX-2 in SW1116 cells when compared with the control group. This induction was associated with the increase in cellular proliferation in the same treatment group. The figure also shows that ethanol extract dose-dependently up-regulated the expression of COX-2 by THP-1 cells, with a significant increase by a high concentration of the extract.

The interaction of ethanol extract with macrophage pre-incubated medium on COX-2 expression by SW1116 cells is illustrated in Fig. 4. The figure shows that incubation with

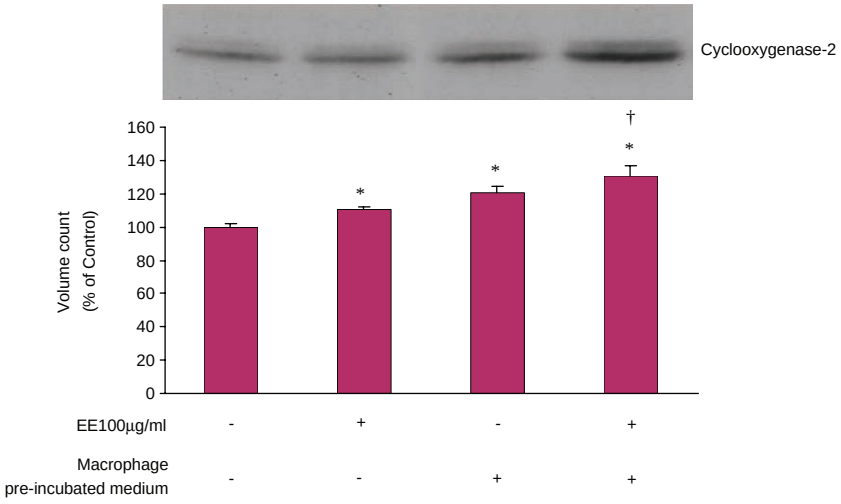

Fig. 4. Cyclooxygenase-2 expression by SW1116 cells after 18 h incubation of cigarette smoke extract (EE) and/or macrophage pre-incubated medium. The medium was derived from macrophages (activated THP-1 cells) after incubation with or without EE for 5 h before incubation with SW1116 cells. Values were expressed as mean ± S.E.M. *$P<0.05$ when compared with the control group. $^{\dagger}P<0.05$ when compared with the EE group.

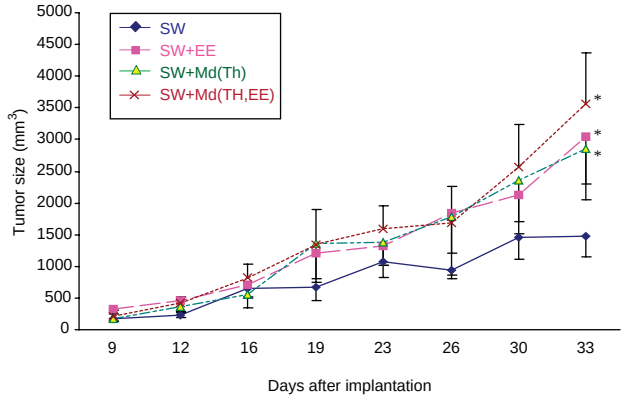

Fig. 5. Tumor size in nude mice after colon cancer cells xenograft implantation. Cells were treated for 18 h before implantation by the following treatments: SW: SW1116 cells only; SW+EE: SW cells treated with cigarette smoke ethanol extract (EE, 100 μg/ml) for 18 h. SW+Md (Th) or SW+Md (Th, EE): SW cells treated with macrophage pre-incubated medium (Md). Md was used to incubate with THP-1 cells (Th) with EE (Md (Th, EE)) or without EE (Md (Th)) for 5 h before incubation with SW1116 cells. Values were expressed as mean±S.E.M. Sample size: 4–8 mice per group. *$P<0.05$ when compared with the SW group.

macrophage pre-incubated medium significantly enhanced COX-2 expression by the colon cancer cells 18 h later. Moreover, the macrophage pre-incubated medium, containing ethanol extract, further induced the level of COX-2 in SW1116 cells. The induction of COX-2 by the combined treatments was significantly higher than the EE incubation alone ($P<0.05$).

3.3. Growth assessment of cancer cell xenograft

SW1116 cells, after treatment with ethanol extract or macrophage pre-incubated medium, with or without ethanol extract, were collected and implanted to nude mice to study cell growth in vivo. Fig. 5 shows the tumor size in nude mice in the 33-day experimental period after the xenograft implantation. SW1116 cells with either ethanol extract treatment or macrophage pre-incubated medium with or without ethanol extract, possessed a gradually larger tumor volume by the end of 33 days, when compared with the SW116 group (without any treatment) ($p<0.05$). Macrophage pre-incubated medium containing ethanol extract produced the biggest response in tumor growth in nude mice.

3.4. Assessment of proliferation, apoptosis and angiogenesis in tumor implant

In the event of increased tumor growth by different treatments, the effects of ethanol extract and macrophage pre-incubated medium on cellular proliferation, apoptosis and angiogenesis were assessed in the tumor implant. Table 1 shows the number of proliferating cells, apoptotic cells and blood vessels in tumor transplants 33 days after implantation. Both ethanol extract and macrophage pre-incubated medium significantly induced cellular proliferation in tumor implants, with the largest increase by the pre-cultured medium containing ethanol extract, when compared with the group with cancer cells without any pretreatment.

However, neither ethanol extract nor macrophage pre-incubated medium treatment alone affected the rate of apoptosis. Only the tumor implants in the group with pre-incubated medium and ethanol extract had a significant reduction in apoptosis when compared with the cancer cell alone group (Table 1). On the other hand, treatment with ethanol extract or macrophage pre-incubated medium increased the number of blood vessels in tumor implants. When the pre-incubated medium contained ethanol extract was applied before implantation, angiogenesis was further enhanced.

Table 1
Assessment of cell proliferation, apoptosis and angiogenesis in tumor implants in nude mice

Groups	Number of proliferating cells per 2 fields	Number of apoptotic cells per 2 fields	Number of blood vessels per mm^2
SW	36.33±2.22	7.50±0.81	3.45±0.32
SW+EE	56.50±1.50[a]	6.93±0.56	4.52±0.41[a]
SW+Md (Th)	45.05±0.087[a]	6.75±0.76	4.28±0.39[a]
SW+Md (Th, EE)	67.20±2.01[b]	5.50±0.84[a]	5.10±0.38[b]

Cells were treated 18 h before implantation by the treatments as described in Fig. 5. Values were expressed as mean±S.E.M. [a]$P<0.05$, [b]$P<0.01$ when compared with the corresponding SW group.

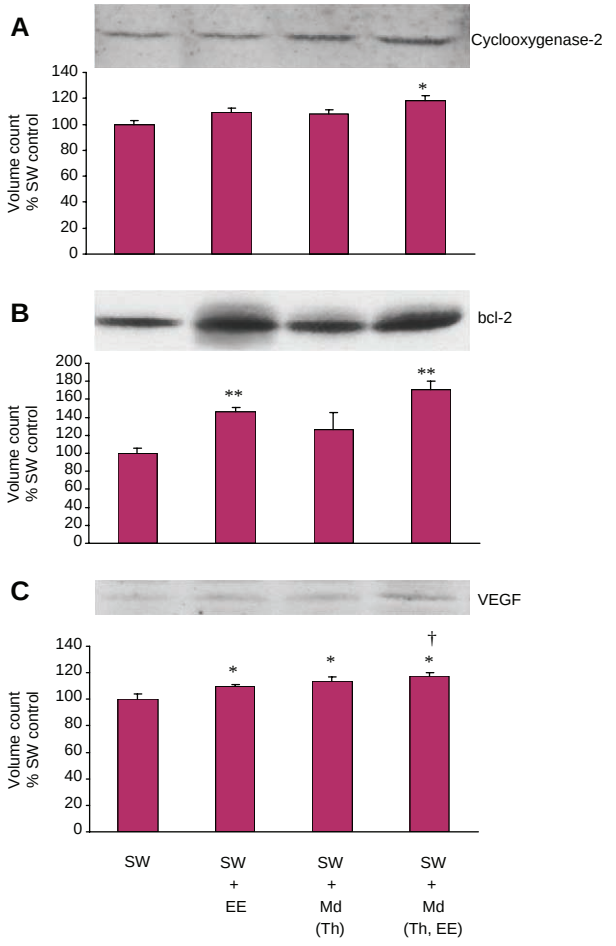

Fig. 6. Expression of (A) cyclooxygenase-2, (B) bcl-2 and (C) vascular endothelial growth factor in tumor implants in nude mice 33 days after xenograft implantation. Cells were treated for 18 h before implantation by the treatments, as described in the Fig. 5. Values were expressed as mean±S.E.M. Sample size: 4–8 mice per group. *$P<0.05$, **$P<0.01$ when compared with the corresponding SW group. †$P<0.05$ when compared with the corresponding EE group.

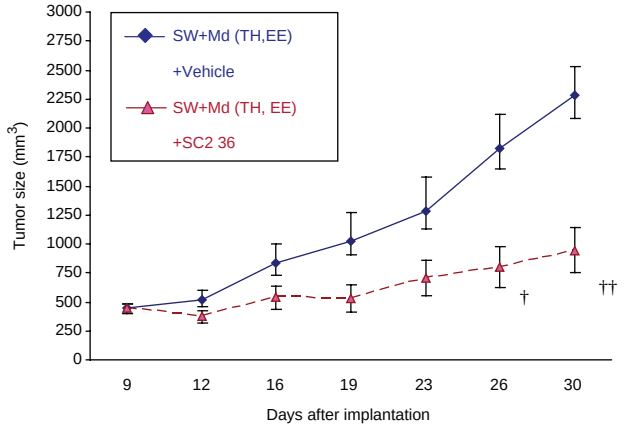

Fig. 7. Effect of SC236 on tumor size in nude mice after colon cancer cells xenograft implantation. SW+Md (Th, EE): SW: SW1116 cells were treated with macrophage pre-incubated medium (Md) for 18 h before implantation. Md was the medium derived from the THP-1 cells (Th) incubated with cigarette smoke ethanol extract (EE, 100 μg/ml) for 5 h. SC236 group: SC236 given twice weekly (2.0 mg/kg, i.p.) following implantation; Vehicle group: SC236 dissolvent without SC236 was given. Values were expressed as mean±S.E.M. Sample size: 6–8 mice per group. $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ when compared with the vehicle group.

3.5. Immunoblotting on protein expression in tumor implants

The levels of COX-2, bcl-2 and VEGF were determined in tumor implants 33 days after implantation and are shown in Fig. 6. COX-2 was slightly altered by either ethanol extract or medium treatment (Fig. 6A). However, combined ethanol extract and medium treatments increased the expression of COX-2 protein, when compared with the SW1116 alone group ($p<0.05$).

The level of bcl-2, an anti-apoptotic protein, is shown in Fig. 6B. The figure shows that a significant induction was found in the ethanol extract treatment group ($p<0.05$) and the medium plus ethanol extract treatment group, with a larger increase in the latter group ($p<0.01$), when compared with the control group. This indicated the macrophage pre-incubated medium with ethanol extract treatment provoked a larger increase in bcl-2 expression.

Fig. 6C shows the level of VEGF in the tumor implant. The level was increased in all the treatment groups ($P<0.05$). Again macrophage pre-incubated medium with ethanol extract further induced the VEGF expression ($P<0.05$) when compared with the ethanol extract group.

Table 2
Effect of SC236 on cell proliferation, apoptosis and angiogenesis in tumor implants in nude mice

Groups	Number of proliferating cells per 2 fields	Number of apoptotic cells per 2 fields	Number of blood vessels per mm^2
SW+Md (Th, EE)+ vehicle	57.50±2.50	5.50±0.78	5.05±0.42
SW+Md (Th, EE)+ SC236	23.22±1.60^{b}	6.52±0.65	3.62±0.38^{a}

SW1116 cells (SW) treated with pre-conditioned medium (Md) for 18 h before implantation. Vehicle and SC236 treatment were given as described in Fig. 7. Values were expressed as mean±S.E.M. Sample size: 6–8 mice. $^{a}P<0.05$, $^{b}P<0.01$ when compared with the vehicle group.

3.6. Effect of COX-2 inhibition on growth of xenograft

The importance of COX inhibition on tumor growth was further studied in the tumor implant in nude mice. Fig. 7 shows the effect of SC236 on tumor volume of the implant in nude mice. SC236 treatment significantly lowered the pace of tumor growth when compared with the vehicle treatment group during the 30-day experimental period ($P<0.05$).

Table 2 shows the effect of SC236 on cell turnover in tumor implants 30 days after cancer cell implantation. SC 236 treatment significantly reduced the cellular proliferation when compared with the vehicle-treatment group ($P<0.01$). This down-regulation of proliferation was associated with a significant reduction of COX-2 protein expression in the same treatment group (Fig. 8A). However, there was a slight but not significant increase in apoptosis in the

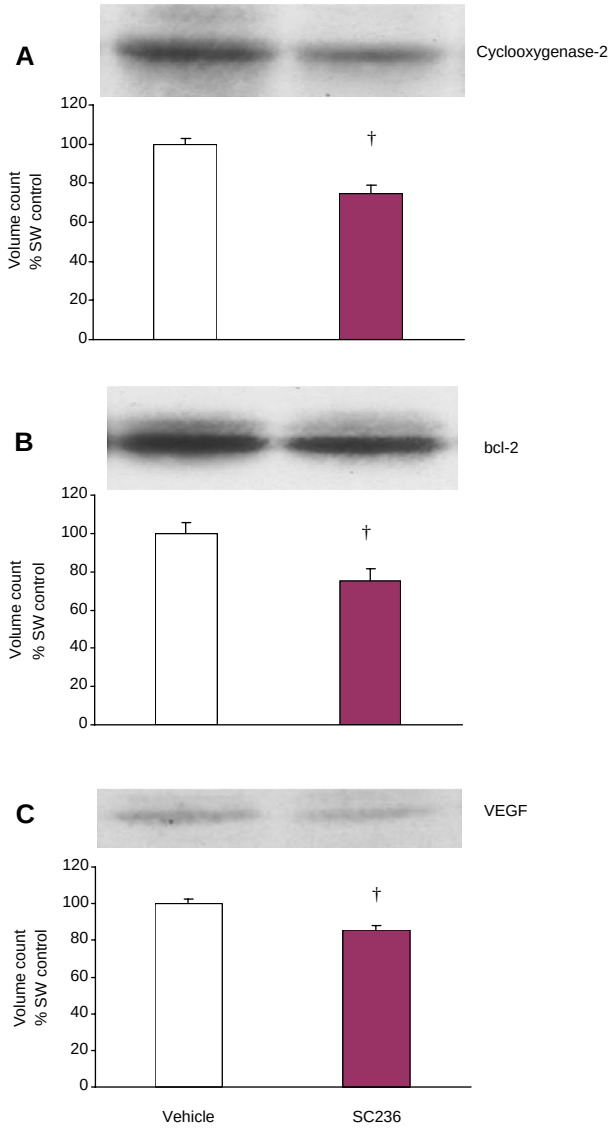

Fig. 8. Effect of SC236 on protein expression of (A) cyclooxygenase-2, (B) bcl-2 and (C) vascular endothelial growth factor in tumor implant in nude mice 1 month after xenograft implantation. Vehicle and SC236 were given to nude mice, as mentioned in Fig. 7. Values were expressed as mean±S.E.M. Sample size: 6–8 mice per group. $^{\dagger}P<0.05$, when compared with the vehicle group.

SC236-treated group. This slight increase was associated with a significant drop of bcl-2 protein expression in the same treatment group (Fig. 8B).

Whether or not SC236 application would affect angiogenesis in tumor implants was also studied. Table 2 reveals a significant decline in number of blood vessels in the SC236 treatment group, when compared with vehicle-treated group ($P<0.05$). This reduction was associated with a significant decrease in VEGF protein expression in tumor implants (Fig. 8C).

4. Discussion

The present study demonstrates the possible involvement of macrophages and contribution of COX-2 in the tumorigenic action of cigarette smoke on colon cancer. Previous study shows that the immunoreactive COX-2 is predominantly and strongly expressed in human sub-epithelial interstitial cells (Bamba et al., 1999). The cell type is further clarified to be macrophages in the human ovarian cancer and Min mouse (Hull et al., 1999; Klimp et al., 2001). Up-regulation of COX-2 expression in lamina propria macrophages may precede the loss of the second functional Apc allele in epithelial cells before adenoma formation in the mouse colon cancer model. This result indicates that COX-2 expression in macrophages could appear early in the colon tumorigenesis. Besides, recent studies also shows that COX-2 is overexpressed by cigarette smoke in fibroblasts (Martey et al., 2004) and in the oral mucosa of active smokers (Moraitis et al., 2005). These findings strongly signify the relationship among cigarette smoke, macrophages and COX-2 in the tumorigenesis of gastrointestinal cancers.

In the present study, ethanol extract induced COX-2 in both adenocarcinoma cells (SW1116) and activated macrophages (THP-1), which was associated with an increase of proliferation of SW1116 cells. Incubating the cells with the medium pre-incubated with activated THP-1 cells, the cell proliferation and COX-2 expression were up-regulated. These findings suggest that the presence of COX-2 or its associated genes released from activated macrophages in the medium could be responsible for the colon cancer cell growth. Moreover, when ethanol extract was added to macrophages first before the medium was used to culture the SW1116 cells, a further increase in both proliferation and COX-2 was observed, implying that the stimulating effects produced by macrophages and ethanol extract on COX-2 expression and cell proliferation were additive.

The effects of ethanol extract and macrophage pre-incubated medium on tumor cell growth were further studied in live animals because the in vivo environment would represent a more realistic condition for tumor growth in situ. Interestingly the tumor volume of the implant was bigger in the ethanol extract and macrophage pre-incubated medium treatment groups. The number of cells injected into nude mice was fixed among different treatment groups to assure any substantial change in tumor growth was only due to the treatments of cancer cells before the implantation into nude mice. In fact, factors responsible for the stimulatory action of ethanol extract and macrophage pre-incubated medium on tumor growth were initiated early before the implantation. It was shown that COX-2 induction has already occurred in colon cancer cells after direct treatment with ethanol extract or the macrophage pre-incubated medium with or without ethanol extract in the cell culture. Such effect was able to initiate a full strength of tumorigenic cascade to promote tumor growth in nude mice. Indeed, previous studies showed that COX-2 expression by adenoma cells was boosted by prostaglandin E_2 through EP_2 receptor via a positive feedback loop (Sonoshita et al., 2001). This self-activation process could also enable tumor cells to produce other pro-carcinogenic factor such as VEGF, basic fibroblast growth factor and matrix metalloproteases (Chambers et al., 1997; Tsujii et al., 1998). In additional to the direct source of COX-2 from the implanted cancer cells, this inducible enzyme can also be derived from neighboring inflammatory cells, including monocytes/macrophages, which could be induced during the implantation of cancer cells in nude mice. The localization of COX-2 in macrophages implies a paracrine action of this inducible enzyme on epithelial cells in adenomas and forms a positive feedback loop of COX-2 expression between epithelial cancer cells and macrophages. All these findings could explain in part why ethanol extract together with the pre-incubated macrophage medium promoted tumor growth in an additive manner, through the enhancement of COX-2 expression in tumor implants.

Previous studies indicate that the major component of cigarette smoke extract, nicotine and its metabolite, could stimulate colon cancer growth, which was also shown to be COX-2 dependent (Ye et al., 2004a,b). Moreover, it has been demonstrated that nicotine can induce mucin synthesis in colon cells (Finnie et al., 1996), suggesting that mucin could also be induced in the colon cancer cells after ethanol extract treatment. Mucins retained in the tumor implant could in turn induce COX-2 expression in monocytes/macrophages (Inaba et al., 2003), thus further establishing the positive feedback loop of COX-2 released from colon cancer cells and neighboring inflammatory cells to promote tumor growth.

The induction of tumor volume by ethanol extract was associated with a significant increase in cell proliferation and angiogenesis, but with a slight reduction of apoptosis, implicating cellular proliferation and angiogenesis by large are the contributory factor to the promotion of tumor growth by ethanol extract in nude mice. The significant up-regulation of COX-2 and VEGF in this group of animals could be responsible in the provocation of cell proliferation and angiogenesis induced by ethanol extract.

Similar findings were observed in the group treated with macrophage pre-incubated medium. This reflects the ability and importance of macrophages to release angiogenic and trophic factors for tumor cell development. Indeed, VEGF

could be produced in macrophages and other stromal cells in response to COX-2 and prostaglandins during tumorigenesis (Bamba et al., 1999; Cheng et al., 1998). Moreover, when colon cancer cells were treated with macrophage pre-incubated medium together with ethanol extract (Th, EE), there was a much larger induction of cellular proliferation and angiogenesis. This was associated with a bigger response in COX-2 and VEGF protein expressions. In addition, there was a significant reduction of apoptosis together with an up-regulation of bcl-2, which had been reported to be strongly associated with the promotion of adenocarcinoma in the gastric mucosa (Lauwers et al., 1995). These findings demonstrate the additive and combined effects of cigarette smoke extract and macrophages on colon tumor growth in mice.

The involvement of COX-2 in the tumor promoting action of ethanol extract was further verified with a COX-2 inhibitor, SC236. The inhibitory action of SC236 on tumor growth was confirmed and this was associated with a significant decrease of proliferation and angiogenesis but with a slight increase in apoptosis in colon tumors. These biological events were accompanied with a significant reduction of bcl-2 and VEGF protein expression in tumor implants, resulting in inhibition of tumor growth. These findings further rectify the importance of COX-2 in the regulation of cell proliferation and apoptosis of colon cancer cells and the generation of blood vessels in tumor implants. Interestingly, COX-2 expression was also down-regulated in tumor implants, following the treatment with SC236. As mentioned above, COX-2 could induce its own expression in a positive feedback mechanism, through its active metabolite prostaglandin E_2 (Sonoshita et al., 2001). SC236 treatment might have blocked the synthesis of prostaglandin E_2 and thereby inhibited the feedback loop on the COX-2 expression in colon cancer cells. The down-regulation of COX-2 could amplify the inhibitory effect of SC236 on prostaglandin synthesis and finally tumor growth. Indeed this is reinforced by the finding that prostaglandin E_2 by itself has a direct stimulatory action on colon carcinoma cells (Kojima et al., 2000).

In conclusion, cigarette smoke extract could promote tumor growth either directly on colon cancer cells or indirectly via macrophages. Both effects could likely be mediated by activation of COX-2 and up-regulation of the expression of bcl-2 and VEGF, resulting in the induction of cellular proliferation and angiogenesis and perhaps also inhibition of apoptosis. All these would favor tumor growth in the colon during cigarette smoking.

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