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A mechanistic study of colon cancer growth promoted by cigarette smoke extract

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Abstract

Substantial evidence indicates that significant exposure to cigarette smoke is associated with an elevated risk for colorectal cancer. However, the mechanisms underlying the causal relationship between cigarette smoking and colorectal cancer remain to be investigated. Our previous study showed that cigarette smoke promotes the formation of inflammation-associated colonic adenoma in mice through an angiogenic pathway. Therefore, in the present study, we used the human colon adenocarcinoma cell line, SW1116, and human umbilical vascular endothelial cells (HUVECs) to elucidate the possible mechanisms in vitro. Results showed that cigarette smoke extract enhanced cell proliferation and the expression of 5-lipoxygenase (5-LOX), vascular endothelium growth factor (VEGF), matrix metalloproteinases (MMPs) 2 and 9 in SW1116 cells. Inhibition of 5-LOX decreased cell proliferation and expressions of VEGF, MMP-2 and MMP-9 induced by cigarette smoke extract. In addition, cigarette smoke extract indirectly stimulated HUVEC proliferation, a biological activity closely related to angiogenesis during tumor growth. This was again blocked by the 5-LOX inhibitor. Taken together, the results of the present study demonstrate the central role of 5-LOX and its relationship with angiogenic mediators in the actions of cigarette smoke in the promotion of angiogenesis during colon cancer growth.

Keywords: 5-LOX; Colon cancer; Cigarette smoke; Angiogenesis; MMP; VEGF1

1. Introduction

Cigarette smoking has been consistently associated with a high risk for colon adenoma and hyperplastic polyp formation (Martinez et al., 1997; Potter et al., 1999), as well as increased incidence of colorectal carcinoma (Newcomb et al., 1995; Hsing et al., 1998; Terry et al., 2001; Limburg et al., 2003). A large-scale clinical trial demonstrated a dose-dependency of smoking on colon adenoma and cancer formation (Giovannucci et al., 1996). Smoking for less than 20 years is associated with an increased risk of small adenoma formation in the colon, while large adenomas are produced in those smoking for more than 20 years. Heavy and long-term cigarette smoking (more than 35 years) leads to a higher odd ratio for colon cancer. The long latency period between the start of smoking and increase in cancer risk, and the significant positive association between smoking and the formation of colon adenoma, reveal that smoking could play a role in colorectal carcinogenesis. Moreover, the risk of death from colorectal cancer is higher in current or former smokers than in non-smokers (Heineman et al., 1994). However, the mechanisms underlying the causal relationship between cigarette smoking and colorectal cancer remain to be clarified.

It is understood that angiogenesis, sprouting of new blood vessels from existing vessels (Hanahan and Weinberg, 2000), is fully responsible for tumor development. Angiogenesis is characterized by a series of steps that includes

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degradation of the basement membrane, endothelial cell proliferation, invasion of the surrounding stroma and structural reorganization into a novel functional vascular network through the recruitment of perivascular supporting cells. This complex process involves multiple regulators such as growth factors, adhesion molecules and matrix-degrading enzymes. Activation of endothelial cells is tightly controlled by a dynamic balance between positive and negative regulators (Hanahan and Folkman, 1996; Witmer et al., 2003). The angiogenic phenotype is now considered to represent a distinct step in the multistep pathogenesis of cancer. This step is known as 'the angiogenic switch', which occurs at the preneoplastic stage of tumorigenesis (Bergers et al., 2000; Folkman, 1996). A plethora of molecules can act as inducers for angiogenesis. They include acidic fibroblast growth factor, basic fibroblast growth factor (bFGF), transforming growth factor alpha and beta (TGF-α and -β), tumor necrosis factor alpha and interleukin-8 (IL-8). However, the major growth factors specific for vascular endothelium are members of the vascular endothelium growth factor (VEGF) (Yancopoulos et al., 2000). Matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) also play important roles in the angiogenic switch (Bergers et al., 2000).

Our previous study showed that cigarette smoke promotes the formation of inflammation-associated colonic adenoma in mice through an angiogenic pathway (Liu et al., 2003; Ye et al., 2004) and 5-LOX plays a central role in this process. In order to further elucidate the angiogenic action of 5-LOX in colon cancer development, we used a human colon adenocarcinoma cell line and human umbilical vascular endothelial cells (HUVECs) to investigate possible mechanisms underlying the induction of angiogenesis by cigarette smoke in vitro.

2. Materials and methods

2.1. Chemicals and drugs

AA861 was purchased from Cayman Chemical (Ann Arbor, MI) and Ro28-2653 was from Roche Diagnostics (Penzberg, Germany). All other chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise specified.

2.2. Cigarette smoke extract preparation

Cigarette smoke extract was prepared by passing non-filtered cigarette smoke through four successive flasks of 95% ethanol followed by two flasks of absolute chloroform (Shin et al., 2002). The ethanol- and chloroform-soluble fractions were concentrated by evaporation and then combined and used as the cigarette smoke extract. This extract contains the major components in the smoke of burning cigarettes (Chow et al., 1997). The nicotine concentrations in this extract ranging from 10–100 μg/ml, were similar to the blood levels in light, moderate and heavy smokers, respectively (Lawson et al., 1998).

2.3. Cell culture

Cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA). HUVECs, human umbilical vascular endothelial cells, were propagated in F-12K medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/ml heparin, and 30 μg/ml bovine endothelial cell growth supplement. SW1116, human colon adenocarcinoma cell line established from a 73-year old man, was cultured in Leibovitz's L-15 medium containing 10% FBS.

2.4. [^{3}H]-thymidine incorporation assay for cell proliferation in culture cells

Cells were seeded into a 24-well plate and cultured for 24 h for attachment. They were then washed twice with 0.01 M phosphate-buffered saline, followed by incubation with 1 ml/well of the medium containing various substances for different time intervals. 0.5 μCi of [^{3}H]-thymidine was added to each well, and cells were further incubated for 5 h. Incorporation of [^{3}H]-thymidine into cells was measured with a liquid scintillation counter (LS-6500; Beckman Instruments, Inc.) (Galffy et al., 1999).

In the non-contact co-culture system, SW1116 and HUVECs were first cultured separately using cell-culture inserts (Becton Dickinson, Tokyo, Japan). The SW1116 cells were plated at the bottom of the culture inserts and the HUVECs at the bottom of a 24-well culture-plate. The cell-culture inserts had a pore size of 0.4 μm and a pore density of 1.6×10^6 pores/cm^2 to allow the bi-directional diffusion of molecules but not the migration of cells. After attachment, cells were washed 3 times with serum-free DMEM/F12 medium, and the culture inserts and the wells were then assembled as shown in Fig. 1. Then, cells were incubated with 2 ml of serum-free DMEM/F-12 medium for 24 h, followed by adding cigarette smoke extract into the culture insert for incubation for another 24 h with or without 1-h pre-treatment with different chemicals. Endothelial cells were then collected for proliferation study.

2.5. Western-blot analysis

Samples were harvested at 4 °C with RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% α-cholate, 0.1% sodium dodecyl sulphate (SDS), 2 mM EDTA, 1% Triton X-100 and 10% glycerol) containing 1.0 mM phenylmethylsulfonyl fluoride and 1

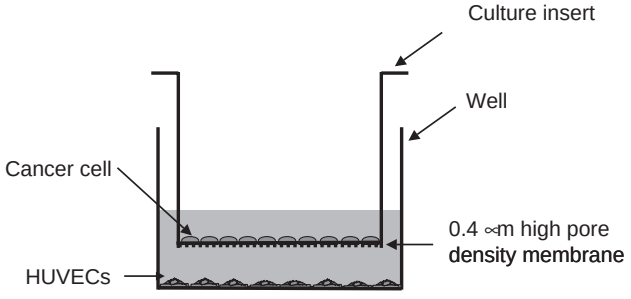

Fig. 1. Non-contact co-culture system. Colon cancer cells and vascular endothelial cells (HUVECs) were co-cultured under non-contact condition. The cancer cells were plated at the bottom of a culture insert and the vascular endothelial cells at the bottom of a culture-plate well. The culture insert was placed in the well and the cells were then incubated.

μg/ml aprotinin (Pai et al., 2002). After sonication on ice, samples were centrifuged at 17,968 ×*g* for 20 min at 4 °C and the supernatant containing 70 μg of protein was denatured and separated by electrophoresis on a SDS polyacrylamide-gel. The protein was then transferred to a nitrocellulose membrane that was probed with respective primary antibody. Membranes were developed in an enhanced chemiluminescence solution and exposed on X-ray film. Quantification of the bands on the film was carried out by video densitometry (Gel Doc 1000, Bio Rad).

2.6. Statistical analysis

Data were expressed as means±S.E.M. (standard error of mean). Student's *t* test was used to compare data between two groups. One-way analysis of variance and Bonferroni correction were used to compare data between three or more groups. *P* values less than 0.05 were considered significant.

3. Results

3.1. Effect of cigarette smoke extract on colon cancer cell proliferation

Since cell proliferation is an important parameter of cancer growth, we investigated whether cigarette smoke extract could promote SW1116 cell proliferation in vitro by the [^{3}H]-thymidine incorporation method. Cigarette smoke extract stimulated SW1116 cell proliferation in a time- and dose-dependent manner. After incubation with 100 μg/ml cigarette smoke extract for 5 h, cell proliferation was increased by about 31% over the control group (Fig. 2).

3.2. Effects of cigarette smoke extract on 5-LOX, MMP-2, MMP-9 and VEGF protein expression in colon cancer cells

We further examined the possible mechanisms involved in the promotion of SW1116 growth by cigarette smoke extract. In this regard, we found that, after incubation with 100 μg/ml cigarette smoke extract for 5 h, the protein expression of 5-LOX, a cell proliferation promoter, was significantly induced in the cells. In addition, this was accompanied by the up-regulation of angiogenic factors MMP-2, MMP-9 and VEGF (Fig. 3).

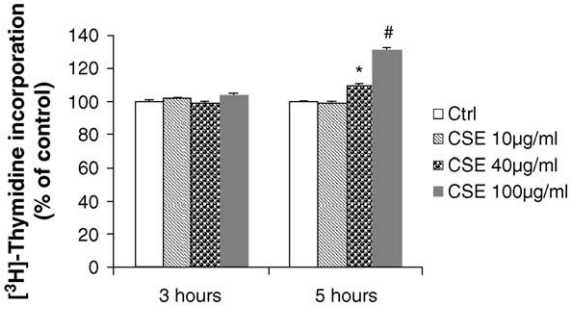

Fig. 2. Effect of cigarette smoke extract (CSE) on SW1116 colon cancer cell proliferation after incubation for 3 and 5 h, at the doses of 10, 40 and 100μg/ml. Data are expressed as mean±S.E.M. of six independent experiments. *$p<0.05$, #$p<0.01$ compared to the corresponding control group (Ctrl).

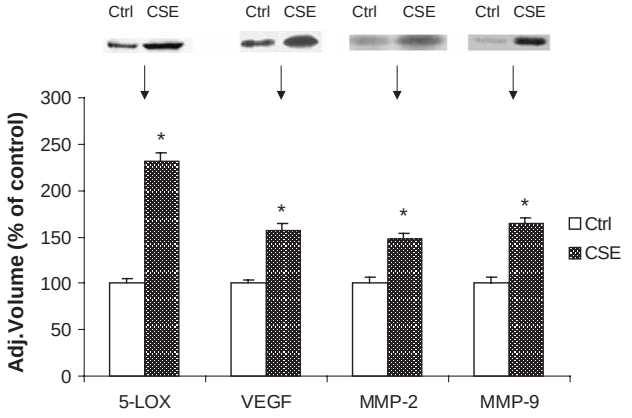

Fig. 3. Effect of cigarette smoke extract (CSE) on 5-LOX, VEGF, MMP-2 and MMP-9 protein expression in SW1116 colon cancer cells. SW1116 cells were treated with 100 μg/ml cigarette smoke extract for 5 h and then collected for assay. Data are expressed as mean±S.E.M. of six independent experiments. *$p<0.05$ compared to the corresponding control group (Ctrl).

3.3. Effect of 5-LOX inhibitor on colon cancer cell proliferation

Since 5-LOX protein expression was significantly enhanced during the process of cell proliferation promoted by cigarette smoke extract, we examined further whether 5-LOX is involved in this process. Result showed that AA861, a specific 5-LOX inhibitor, dose-dependently and partially blocked the cell proliferation induced by cigarette smoke extract (Fig. 4).

3.4. Effects of 5-LOX inhibitor on MMP-2, MMP-9 and VEGF protein expressions in colon cancer cells

To examine the relationship between 5-LOX and the angiogenic factors, SW1116 cells were pre-treated with 10 or 20 μM AA861 for 1 h before the addition of cigarette smoke extract. The inhibitor significantly impeded the protein expressions of MMP-2, MMP-9 and VEGF induced by cigarette smoke extract. However, the two

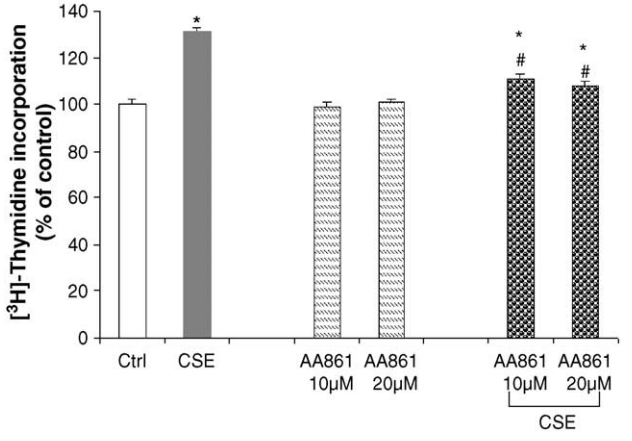

Fig. 4. Effect of 5-LOX inhibitor (AA861) on SW1116 cell proliferation induced by cigarette smoke extract (CSE). SW1116 cells were pre-treated with AA861 at 10 or 20 μM for 1 h before the administration of 100 μg/ml cigarette smoke extract for further 5-h incubation. Data are expressed as mean±S.E.M. of six independent experiments. *$p<0.05$ compared with the control group; #$p<0.05$ compared with the cigarette smoke extract-treated group.

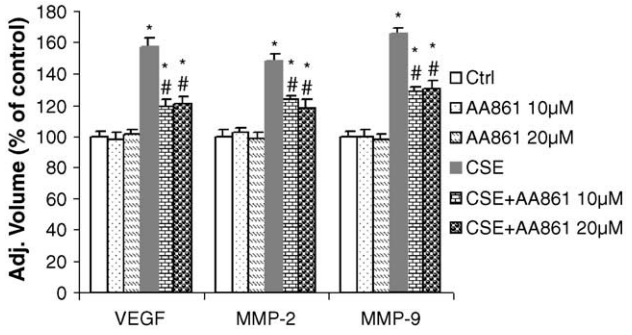

Fig. 5. Effect of 5-LOX inhibitor (AA861) on the protein expressions of VEGF, MMP-2 and MMP-9 induced by cigarette smoke extract (CSE). SW1116 cells were pre-treated with AA861 at 10 or 20 μM for 1 h before the administration of 100 μg/ml cigarette smoke extract for further 5-h incubation. Data are expressed as mean±S.E.M. of six independent experiments. *$p<0.05$ compared with the control group; #$p<0.05$ compared with the cigarette smoke extract-treated group.

doses of AA861 appeared to produce similar and partial inhibitory action on the protein expression. Further reduction was not found at the higher dose (Fig. 5).

3.5. Effect of MMP inhibitor on VEGF expression in colon cancer cells

The above results suggest that MMP-2, MMP-9 and VEGF are down-stream mediators of the 5-LOX-mediated signal pathway in the induction of angiogenesis in colon cancer growth. However, the relationship between MMP and VEGF remains undefined. In this context, we pre-treated colon cancer cells with MMP-2 and -9 inhibitor, Ro28-2653, at doses of 0.1 and 1 μM for 1 h before the administration of cigarette smoke extract. Results showed that the MMP inhibitor alone at these concentrations did not affect the basal level of VEGF in colon cancer cells, but it significantly reduced the increased protein expression of VEGF induced by cigarette smoke extract (Fig. 6). The 5-LOX protein expression was also unaffected by this inhibitor. Furthermore, we found that VEGF antibody had no direct effect on the levels of 5-LOX, MMP-2 and MMP-9 induced by cigarette smoke extract (data not shown).

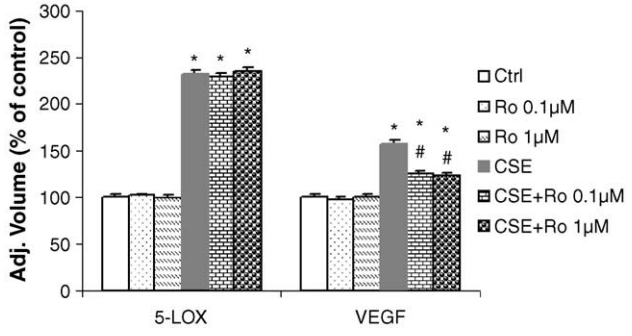

Fig. 6. Effect of MMP inhibitor (Ro28-2653) on 5-LOX and VEGF protein expression in SW1116 cells. SW1116 cells were pre-treated with Ro-28-2653 (Ro) at of 0.1 or 1 μM for 1 h before the administration of 100 μg/ml cigarette smoke extract for further 5-h incubation. Data are expressed as mean±S.E.M. of six independent experiments. *$p<0.05$ compared with the control group; #$p<0.05$ compared with the cigarette smoke extract-treated group.

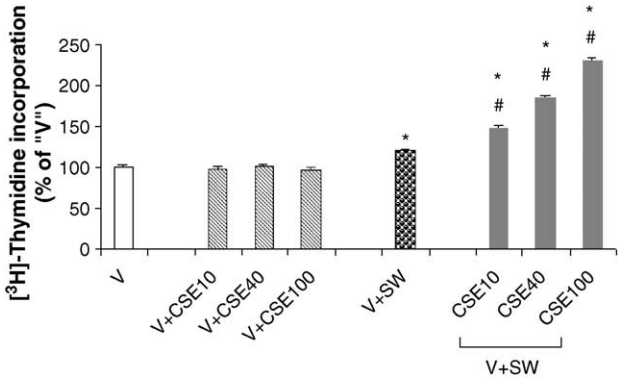

Fig. 7. Effect of cigarette smoke extract (CSE) on [^{3}H]-thymidine incorporation in HUVECs co-cultured with SW1116 cells. V: single-cultured HUVECs group; V+SW: HUVECs co-cultured with SW1116 cells; V+CSE: single-cultured HUVECs treated with different concentrations of cigarette smoke extract (μg/ml) for 24 h; V+SW+CSE: HUVECs co-cultured with SW1116 cells which were treated with different concentrations of cigarette smoke extract (μg/ml) for 24 h. Data are expressed as mean±S.E.M. of six independent experiments. *$p<0.05$ compared to the single-cultured HUVECs group (V), #$p<0.05$ compared to the V+SW group.

3.6. Effect of cigarette smoke extract on cell proliferation of vascular endothelial cells co-cultured with colon cancer cells

Since cigarette smoke extract enhanced the expression of angiogenic factors by colon cancer cells, we investigated whether this action could affect proliferation of vascular endothelial cells. The results showed that cigarette smoke extract stimulated cell proliferation of HUVECs when co-cultured with SW1116 cells (Fig. 7). In the co-culture group (V+SW), [^{3}H]-thymidine incorporation in HUVECs was significantly higher than that in the single-cultured group (V) after 24 h of incubation. Cigarette smoke extract incubation of the colon cancer cells further enhanced this effect in a dose-dependent manner, with a maximum effect at

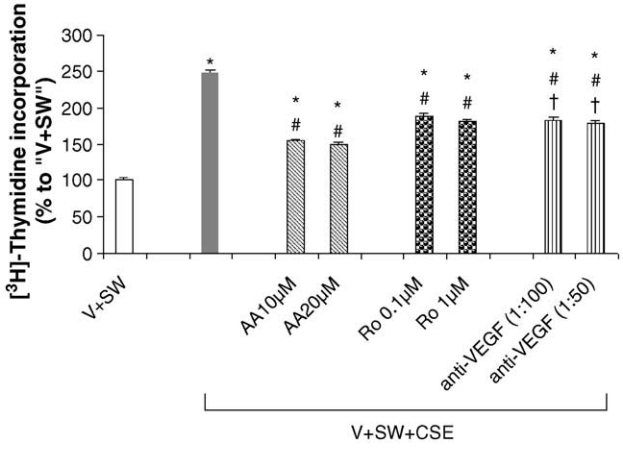

Fig. 8. Effect of 5-LOX inhibitor (AA861: AA), MMP inhibitor (Ro28-2653: Ro) and VEGF antibody (anti-VEGF) on [^{3}H]-thymidine incorporation in HUVECs co-cultured with SW1116 cells. Data are expressed as mean±S.E.M. of six independent experiments. *$p<0.05$ compared to the V+SW group, #$p<0.05$ when compared to the V+SW+CSE group, †$p<0.05$ compared to the AA10 μM+V+SW+CSE group.

the dose of 100 μg/ml. However, cigarette smoke extract had no direct effect on the cell proliferation of HUVECs.

3.7. Effects of 5-LOX inhibitor, MMP inhibitor and VEGF antibody on DNA synthesis in vascular endothelial cells co-cultured with colon cancer cells

To determine if VEGF was the activating factor for HUVECs proliferation, the medium conditioned by colon cancer cells was incubated with the anti-VEGF antibody. Result showed that VEGF antibody (1 : 100 ~ 1 : 50) partially inhibited the stimulatory action of cigarette smoke extract on HUVEC proliferation. Similar results were found with the MMP inhibitor (Ro28-2653) and 5-LOX inhibitor (AA861) (Fig. 8). But AA861 and VEGF antibody had no direct effect on endothelial cell proliferation (data not shown).

4. Discussion

Uncontrolled tumor cell proliferation and new blood vessel formation (angiogenesis) are two major targets in the current cancer therapeutics of cancer. In most forms of solid tumor, genetic and epigenetic alterations are initially responsible for deregulated tumor cell expansion and selection (Hanahan and Weinberg, 2000; Lengauer et al., 1998). After the tumor reaches a certain size, autocrine and paracrine signaling between various growth factors and endothelial receptors are responsible for endothelial cell survival, proliferation, differentiation, and finally angiogenesis in order to facilitate tumor growth (Hanahan and Folkman, 1996; Saaristo et al., 2000).

Treatment for cancer is now moving beyond traditional chemotherapy with the advent of specific targeted therapies, and much research effort is focused on developing treatments based on the inhibition of tumor angiogenesis. It has been shown that neovascularization not only permits further growth of the primary tumor, but also provides a pathway for migrating tumor cells to gain access to the systemic circulation and establish distant metastases.

Given its pivotal role in growth and survival, the tumor vasculature is an attractive target for anticancer therapy. Two key approaches to targeting the tumor blood vessel network have been developed. The first aims to inhibit the tumor-initiated angiogenic process itself. The second involves the use of therapeutic agents to preferentially destroy the established tumor vessel network (Ziche et al., 2004).

Endothelial cells present a preferential target for anti-tumor therapy, as this cell type is common to all solid tumors. Although each type of cancer is virtually a unique disease, tumor endothelium is a relatively uniform and normal tissue (Ferrara and Alitalo, 1999; Folkman et al., 1971). Another advantage of making endothelial cells the anti-angiogenic target of cancer therapy is their apparent inability to counteract therapy through development of multi-drug resistance, due to the low mutagenesis rate of this cell type (Ferrara, 2002).

To provide further evidence that the effect of cigarette smoke on tumorigenesis is through activation of angiogenesis, a co-culture system of human colon cancer cells with endothelial cells was developed in the current study. Our results showed that cigarette smoke extract stimulated SW1116 colon cancer cells to express 5-LOX, VEGF, MMP-2 and MMP-9 proteins, while 5-LOX inhibitor down-regulated their expressions. Furthermore, a MMP inhibitor partially reduced VEGF protein expression induced by cigarette smoke extract, suggesting that MMP is upstream from VEGF in the induction of angiogenesis in the colon.

In the non-contact co-culture system, addition of cigarette smoke extract into the culture insert with colon cancer cells significantly stimulated the proliferation of vascular endothelial cells. However, the extract had no direct effect on the proliferation of HUVECs, suggesting that cigarette smoke extract acts indirectly through the colon cancer cells to release angiogenic factors and subsequently stimulate endothelial cell growth. To confirm this phenomenon, we applied 5-LOX and MMP inhibitors as well as VEGF antibody in the non-contact co-culture system. All these agents partially decrease the vascular endothelial cell proliferation, with a maximum effect in the group treated with the 5-LOX inhibitor. These inhibitors, however, only partially blocked the stimulatory action of cigarette smoke extract on angiogenesis implying that other angiogenic factors could be involved. Indeed, it is generally accepted that cancer cells can produce other angiogenic factors such as bFGF, IL-8 and TGF-β, all of which can cause endothelial cell recruitment and proliferation (Folkman, 1971, 1995; Kerbel, 2000). The relationships among these angiogenic factors and 5-LOX remain undefined. They could also contribute to the angiogenic process in the colon and promote tumor growth by cigarette smoke.

In conclusion, 5-LOX plays a central role in the promotion of cancer growth in the colon and its action is stimulated by cigarette smoke through the activation of cell proliferation and angiogenesis.

Acknowledgements

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