

Upregulation of vascular endothelial growth factor by hydrogen peroxide in human colon cancer

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Abstract

AIM: To evaluate the effect of reactive oxygen species such as hydrogen peroxide on the progression of human colon cancer.

METHODS: Human colon carcinoma cell lines, LS174T and HCT8, were treated respectively with 10^{-5} , 10^{-7} or 10^{-9} mol·L⁻¹ hydrogen peroxide for 24h, and co-cultured with human endothelial cell line ECV-304. The migration of ECV-304 induced by cancer cells was calculated and the expression level of vascular endothelial growth factor in cancer cells was determined by RT-PCR analysis and ELISA. Dactinomycin of 1.5mg·L⁻¹ which could block transcription of cancer cells was applied to observing the effects of H₂O₂ on transcriptional activity and the relative half-life of VEGF mRNA. Finally, to evaluate the effect of H₂O₂ on NF-κB activity in colon cancer cells, NF-κB in cytoplasm and nucleus of the cells were detected with FITC-tagged antibody and its presence in the nucleus(Fn) vs cytoplasm(Fc) was monitored by measuring the green fluorescence integrated over the nucleus by laser scanning cytometry(LSC).

RESULTS: Exogenous hydrogen peroxide of low concentration increased the migration of endothelial cell induced by colon cancer cells. When cancer cells were treated with 10^{-5} mol·L⁻¹ H₂O₂, the migration number of endothelial cells induced by LS174T cells was 203 ± 70 , and the number induced by HCT8 cells was 145 ± 65 . The two values were significantly higher than those treated with other concentrations of H₂O₂ ($P < 0.01$). The expression of vascular endothelial growth factor in cancer cells, which could be blocked by dactinomycin, were increased to a certain degree, while the relative half-life of VEGF mRNA was not prolonged after treatment with hydrogen peroxide. The activity of NF-κB in colon cells rose after the cells were exposed to hydrogen peroxide for 24h. The Fn values in HCT8 cells were 91 ± 13 (0 mol·L⁻¹ H₂O₂) and 149 ± 40 (10^{-5} mol·L⁻¹ H₂O₂) ($P < 0.05$), in LS174T cells were 127 ± 35 (0 mol·L⁻¹ H₂O₂) and 192 ± 11 (10^{-5} mol·L⁻¹ H₂O₂) ($P < 0.05$). It is similar to the case of VEGF expression in cancer cells.

CONCLUSION: Hydrogen peroxide increases vascular endothelial growth factor expression in colon cancer cells, and it is likely that reactive oxygen species such as hydrogen peroxide facilitates the development of colon cancer.

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INTRODUCTION

Reactive oxygen species (ROS) can be easily produced in intracolonic cavity, due to large amounts of bacteria and dietary metabolites in it. Several reports on the relationship between ROS and cancer suggested that ROS such as oxygen radicals, hydroxyl radicals and hydrogen peroxide (H₂O₂), were involved in the pathogenesis of colon tumors^[1-14]. H₂O₂, a special intermediate in redox reaction, is able to cross cell membranes in a free manner and modify protein and nucleic acid after being changed into radicals, and now it is thought to be a kind of signal molecular which plays an important role in the growth of tumor cells. Evidences have been given in some reports that H₂O₂ could promote cell growth and related gene expression in human tumors such as prostate cancer and breast cancer. Considering the special environment in colon and rectum we think it is necessary to evaluate the effects of ROS, especially H₂O₂, on the progression of colorectal cancer^[15-27]. As angiogenesis, which induced by several factors such as vascular endothelial growth factor(VEGF), is often demonstrated in solid tumors, and thought to be an essential requirement for the development of malignant tumors^[28-33], we investigated the effects of H₂O₂ on VEGF expression in colon cancers in this study to find evidences that ROS such as H₂O₂ plays a role in the progression of the tumor.

MATERIALS AND METHODS

Cell culture and culture conditions

Human colon cancer cell lines, LS174T and HCT8, and human umbilical vein endothelial cell line ECV-304 were purchased from American Type Culture Collection. These cell lines were cultured and maintained in RPMI1640 supplemented with 100 ml·L⁻¹ fetal bovine serum at 37°C in 50 ml·L⁻¹ carbon dioxide and 950 ml·L⁻¹ air.

H₂O₂ treatment of cancer cells and MTT assay

To determine the effects of H₂O₂ on the growth of cancer cells, LS174T and HCT8 cells were grown (10^3 per well) on 96-well plates and treated with the culture media containing H₂O₂ (ten concentrations from 10^{-10} mol·L⁻¹ to 10^{-1} mol·L⁻¹) (300 ml·L⁻¹ H₂O₂ solution was purchased from Sigma). After 48h, MTT assay showed that H₂O₂ inhibited the growth of cancer cells when its concentration was $>10^{-5}$ mol·L⁻¹, while had no effect on cell growth when its concentration was $\leq 10^{-5}$ mol·L⁻¹. Therefore, those concentrations of H₂O₂, 10^{-5} , 10^{-7} and 10^{-9} mol·L⁻¹, were used in subsequent studies.

Endothelial cell migration induced by cancer cells

To clarify the effects of H₂O₂ on the migration of vascular endothelial cells, which could be promoted by VEGF, colon cancer cells were co-cultured with endothelial cells. LS174T and HCT8 cells were plated on 12-well plates (Falcon) at a density of 4×10^5 per well. Four hours later, the cells were washed with serum-free medium, and

exposed to 10^{-5} , 10^{-7} or 10^{-9} mol·L⁻¹H₂O₂ with the H₂O₂-containing complete media for 24h. Cell culture inserts with polyethylene terephthalate membranes(PET) and 8 μm pore size (Becton Dickinson, USA) were then placed into the 12-well plates, and endothelial cells ECV-304 were seeded into the inserts with a density of 1×10^4 . Six hours later, the cells on the upper surface of the PET membranes were wiped off completely, and the inserts were fixed and stained. The migration capacity of endothelial cells was estimated by counting the number of the cells beneath the PET membranes. The controls were the groups without cancer cells in the wells or without treating cancer cells with H₂O₂.

Expression of VEGF in colon cancer cell

To determine the effects of H₂O₂ on expression of VEGF, LS174T and HCT8 were grown to 90% confluence to avoid the effects of cell density and incubated in complete media in the presence of H₂O₂ (10^{-5} , 10^{-7} and 10^{-9} mol·L⁻¹) for 24h. Total RNA was extracted and resuspended in sterile RNase-free water for storage at -70°C. Access RT-PCR system (Promega) with the sensitive feature was used to determine the relative VEGF mRNA expression. All primers were synthesized by Life Technology, Hongkong.

VEGF sense: 5'-AAGCCATCCTGTGTGCCCTG ATG-3'
antisense: 5'-GCGAATTCCTCTGCCCGCGCTGAC-3'
β-actin sense: 5'-AACACCCAGCCATGTACGTTG-3'
antisense: 5'-CGGATGTCCACGTCACACTTCAT-3'

The 50 μL mixture for reverse transcription and PCR amplification were added in one-tube including AMV reverse transcriptase 5U, Tfl DNA polymerase 5U, MgSO₄, 20 μL dNTP mixture, reaction buffer and 20 pmol of each primer and 0.1 μg total RNA sample. The condition for RT-PCR included a 48°C reverse transcription, a 94°C AMV inactivation and denaturation, a 60°C annealing and a 72°C extension. PCR amplification was subjected to 40 cycles. A volume of 10 μL RT-PCR products was added in 20g·L⁻¹ agarose gel containing 0.5mg·L⁻¹ EB. After electrophoresis, the density and area of each band were measured using Fluro-sTM image software (Bio-Rad, USA). The relative RNA level of VEGF in tumor cells was calculated using the house-keeping gene β-actin as an internal control. The experiments were repeated at least four times.

Analysis VEGF transcriptional activity and VEGF mRNA half-life

To confirm that the effect of H₂O₂ on expression of VEGF in colon cancer cells was due to an increase in transcription, transcription activity of cancer cells was blocked by Dactinomycin (ActD, purchased from Sigma). LS174T cells were incubated in the presence of ActD ($1.5 \text{mg} \cdot \text{L}^{-1}$) for 4h before their exposure to 10^{-5} mol/L H₂O₂ in serum-free medium. Total RNA was extracted from cells after 24h, and RT-PCR analysis was made. Control cells were treated with ActD without H₂O₂.

To determine the effect of H₂O₂ on VEGF mRNA stability, LS174T cells were incubated in the presence or absence of 10^{-5} mol·L⁻¹ H₂O₂ for 24h. Further transcription in cells was then blocked by addition of $1.5 \text{mg} \cdot \text{L}^{-1}$ ActD. Total RNA was extracted from cells at 0, 0.25, 0.5, 1, 2 and 4h. RT-PCR analysis was made and the relative level of VEGF mRNA expression at each point was compared with the control value (total RNA extracted from cells before ActD treatment was defined as 100%). The relative half-life of VEGF mRNA was determined by plotting relative VEGF mRNA expression levels on a semilogarithmic axis versus time.

Determination of VEGF protein levels

The VEGF protein levels in the supernatant were

determined with an enzyme-linked immunosorbent assay (ELISA) kit. Examinations were repeated three times.

Activity of transcriptional factor NF-κB in colon cancer cells

To evaluate the effects H₂O₂ on NF-κB activity in colon cancer cells, NF-κB in cytoplasm and nucleus of the cells was detected with FITC-tagged antibody and its presence in the nucleus vs cytoplasm was monitored by measuring the green fluorescence integrated over the nucleus by laser scanning cytometry(LSC) according to the Deptala's report. Briefly, the cells were first attached to the microscope slides, and exposed to 10^{-5} mol·L⁻¹ H₂O₂ for 0.5, 1, 3, 6, 12 and 24h. The cells on slides were fixed and incubated with NF-κB P65 antibody (Santa Cruz) and FITC-tagged goat-antirabbit Ig (Santa Cruz) at room temperature. Cellular DNA was then counterstained by addition of a solution containing propidium iodide and RNase (Sigma). The cells were placed on microscope slides mounted under coverslips and analyzed by LSC. At least 10^3 cells were analyzed by LSC per slide. Fluorescence intensity in nucleus (Fn) and in cytoplasm (Fc) were detected and the activity of NF-κB was detected by estimating the value of Fn or Fn/Fc.

Statistics

When appropriate, data were expressed as $\bar{x} \pm s$. Analysis of variance and *t* test were applied to assess the significance of differences. Statistical significance was accepted at $P < 0.05$.

RESULTS

H₂O₂ promotes the migration of endothelial cells induced by colon cancer cells

The migration of endothelial cells induced by cancer cells was promoted to a certain degree when LS174T and HCT8 cells were exposed to 10^{-5} , 10^{-7} or 10^{-9} mol·L⁻¹ H₂O₂ for 24h. When cancer cells were treated with 10^{-5} mol·L⁻¹ H₂O₂, the migration number of endothelial cells induced by LS174T cells was 203 ± 70 , and the number induced by HCT8 cells was 145 ± 65 . The two values were significantly higher than those treated with other concentrations of H₂O₂ (Table 1, Figure 1). When there was no cancer cell in the co-culture system, the number of random motility of endothelial cells was about ten cells.

Upregulation of VEGF expression by H₂O₂

Electrophoresis of RT-PCR products showed the three positive bands, 243, 375 and 509bp, representing VEGF121, VEGF165 and β-actin respectively. The internal control demonstrated a stable expression in colon cells treated with each dose of H₂O₂. The analysis of electrophoresis showed that expression levels of VEGF were elevated in LS174T and HCT8 cells with H₂O₂ exposure, especially with 10^{-5} mol·L⁻¹ H₂O₂ (Figure 2). After inhibition of transcriptional activity by ActD before addition of H₂O₂, induction of VEGF mRNA expression was completely inhibited in LS174T cells (Figure 3), indicating that H₂O₂-induced VEGF expression possibly occurred at the transcriptional level. The relative half-life of VEGF mRNA in LS174T cells treated with H₂O₂ was similar to that of the cells without H₂O₂ exposure, demonstrating that the stability of VEGF mRNA was not affected by the treatment with H₂O₂ (Figure 4). To determine whether secretion of VEGF is increased by H₂O₂ in colon cancer cells, the supernatant was assayed and the results showed that H₂O₂ also promoted the VEGF protein expression. The levels of VEGF protein peaked when LS174T and HCT8 cells were incubated in the media containing 10^{-5} mol·L⁻¹ H₂O₂. This situation is similar to the increase of VEGF expression.

Table 1 The migration of endothelial cells induced by colon cancer cells ($\bar{x}\pm s$)

Cell line	H ₂ O ₂ in media(mol·L ⁻¹)			
	0	10 ⁻⁹	10 ⁻⁷	10 ⁻⁵
LS174T	155±38	162±38	174±40	202±70 ^a
HCT8	113±73	114±71	122±68	145±65 ^b

^aP<0.01, *t*=3.4751, vs LS174T, without H₂O₂ treatment ^bP<0.01, *t*=3.4183, vs HCT8, without H₂O₂ treatment

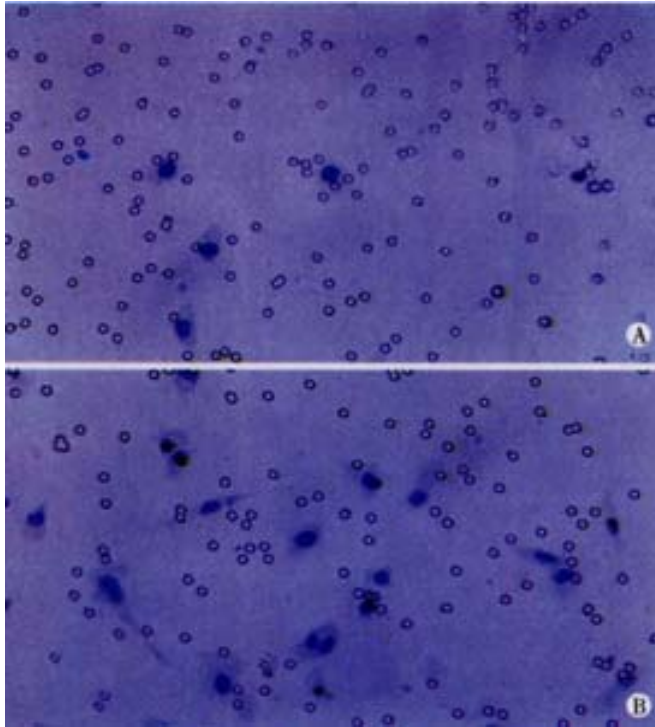


Figure 1 The migration of endothelial cells induced by LS174T cells was increased after the cancer cells were exposed to 10⁻⁵mol·L⁻¹ H₂O₂. The regular circles is the 8µm pores located in PET membrane. A: induced by cancer cells without H₂O₂ treatment. B: induced by cancer cells with H₂O₂ treatment. ×200

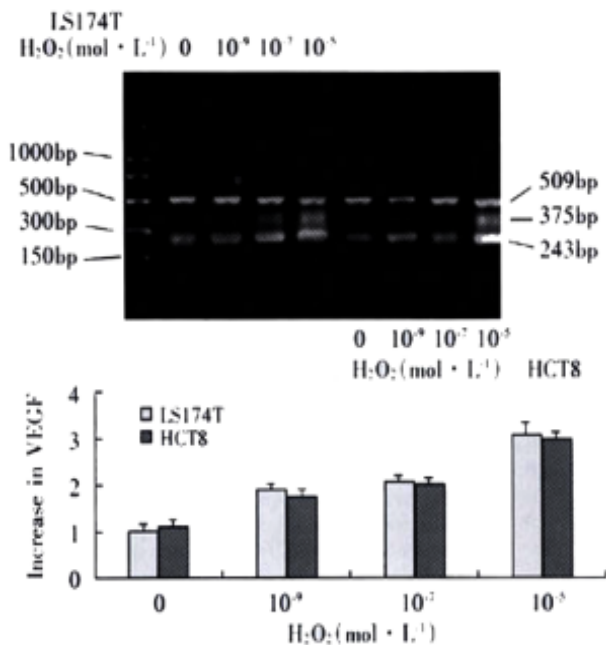


Figure 2 Expression levels of VEGF were increased in LS174T and HCT8 after exposure to 10⁻⁹,10⁻⁷and 10⁻⁵mol·L⁻¹ H₂O₂, demonstrating a dose-dependent feature.

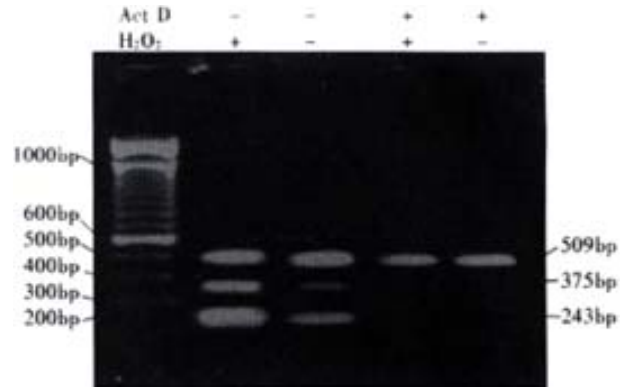


Figure 3 LS174T cells were treated with 1.5mg·L⁻¹ Act D for 4h, followed by exposure to 10⁻⁵ mol·L⁻¹ H₂O₂ for 24h. RT-PCR was done. The control was those without Act D treatment.

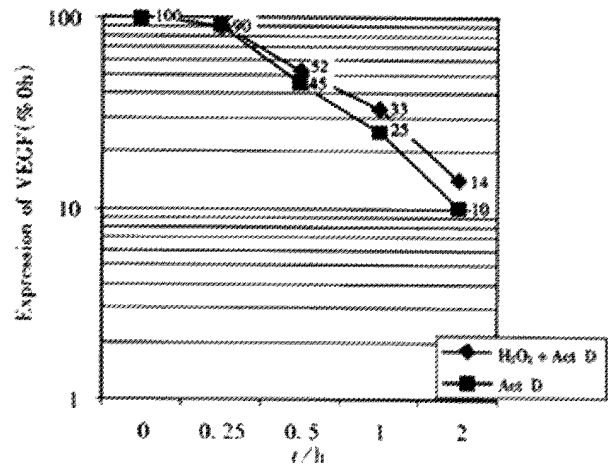


Figure 4 Effect of H₂O₂ on the half-life of VEGF mRNA in LS174T cells with or without H₂O₂ exposure following Act D treatment. No difference in half-life was showed between the two groups.

H₂O₂ increases the activity of NF-κB

LS174T and HCT8 cells were incubated in the presence of H₂O₂ (10⁻⁵mol·L⁻¹) for 0.5,1,3,6,12 and 24h in complete medium. NF-κB activity peaked after exposure to H₂O₂ for 24h. We investigated the changes of NF-κB activity in LS174T and HCT8 cells treated with 10⁻⁵, 10⁻⁷and 10⁻⁹ mol·L⁻¹H₂O₂ in media. Compared with the cells without H₂O₂ treatment, administration of 10⁻⁵ mol·L⁻¹ H₂O₂ for 24h led to a more remarkable increase in green fluorescence intensity measured over nuclear area (Fn) (Table 2,Figure 5), indicating the increase of NF-κB activity in LS174T and HCT8 cells.

Table 2 Change of NF-κB activity in colon cancel cells ($\bar{x}\pm s$)

Cell line	H ₂ O ₂ (mol·L ⁻¹)	
	0	10 ⁻⁵
HCT8 Fn	91±13	149±40 ^a
Fn/Fc	0.75±0.14	2.18±0.54
LS174T Fn	127±35	192±11 ^b
Fn/Fc	2.18±1.17	3.99±1.38

^aP<0.05, *t*=3.4179, vs HCT8, no H₂O₂ treatment

^bP<0.05, *t*=3.0981, vs LS174T, no H₂O₂ treatment

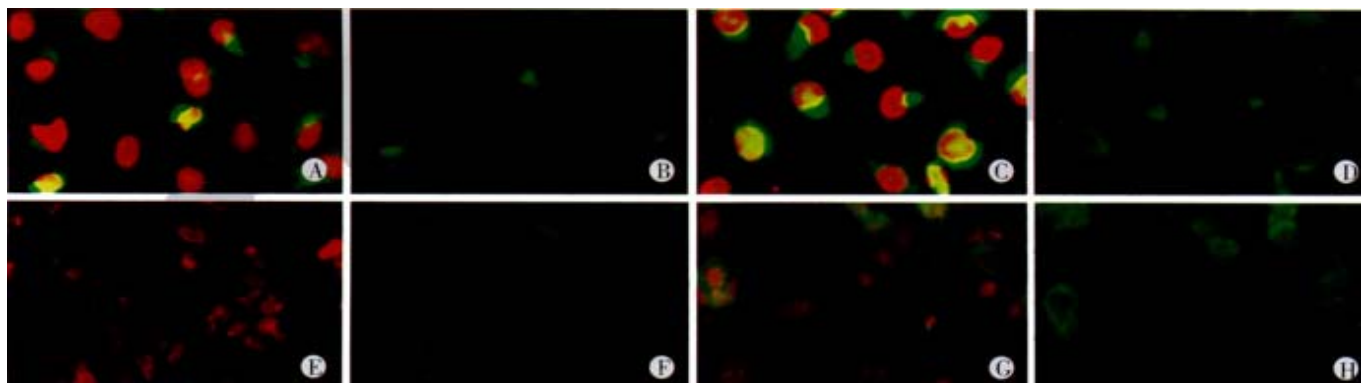


Figure 5 Measurement of nuclear and cytoplasmic NF- κ B associated fluorescence by LSC. The red fluorescence represents the nuclear area, and the intensity of green fluorescence over nucleus(Fn) reflects NF- κ B activity. A, B: LS174T cells without H₂O₂ treatment. C, D: Increase in NF- κ B activity of LS174T cells with H₂O₂ exposure. E, F: HCT8 cells without H₂O₂ treatment. G, H: Increase in NF- κ B activity of LS174T cells with H₂O₂ exposure.

DISCUSSION

The reactive oxygen species(ROS), which are ubiquitous and occur naturally in all aerobic species, may be divided into two categories :free oxygen radicals (\cdot OH, \cdot NO and O₂⁻) and nonradical ROS such as H₂O₂. For decades, H₂O₂ has been one of the ROS that has been well investigated in flammatory response and oxidant-induced stress. Recently, numerous evidence has been presented to show that H₂O₂ can act as a signaling molecule involved in many cellular function such as apoptosis and proliferation^[1-10].And the regulation of series of genes involved in carcinogenesis and progression is associated with the function of H₂O₂^[3,5,6,9,11-14].Several reports have suggested that ROS such as H₂O₂ plays a role in the pathogenesis of tumor in colon, where there are a great deal of bacteria and dietary metabolites^[15-27]. Diet rich in fat increased the formation of ROS in feces, which then possibly damaged the stem cells in the colon^[20,22,26]. However, up to date, little information has been available about the role of H₂O₂ the special reactive oxygen intermediate, in the biological behaviors of colon cancer cells.

VEGF is a potent and unique angiogenic protein that stimulates capillary formation and has specific mitogenic and chemotactic activity for vascular endothelial cells^[28].In colon cancer, VEGF levels are elevated and correlated with a poor clinical outcome^[29-33]. VEGF expression is regulated by some pathological processes such as hypoxia^[34-36]and by numerous cytokines and growth factors including interleukin1 β , interleukin 6, platelet-derived growth factor, transforming growth factor β , epidermal growth factor, hepatocyte growth factor, insulin-like growth factor, angiotensin II, hypoxia -inducible factor I and EIF4E etc.^[37-52] Recently, oncogene p53 is also found to be a regulator of VEGF gene in colon cancer cells^[53,54].In the present study, we found, to our knowledge, for the first time, that exogenous H₂O₂ could up-regulate the expression of VEGF in human colon cancer cells and the migration of endothelial cells induced by the cancer cells after we reviewed those results from RT-PCR assay, ELISA and migration experiment of endothelial cells. Considering the important role of VEGF in neovascularization in solid tumors, we believe that hydrogen peroxide may have the promoting effects on the progression of colon cancer. Related studies also found that hydrogen peroxide could not only increase the expression of VEGF in cultured human vascular smooth muscle cell, human retinal pigment epithelial, melanoma cells and glioblastoma cells, but also promote the growth of prostate and breast cancer cells^[55-58].

NF- κ B activation, as expressed by its translocation from the cytoplasm to nucleus, can be conveniently assayed by LSC by measuring the intensity of NF- κ B-associated immunofluorescence over the area of cell nucleus and comparing it with the intensity over cytoplasm^[59]. NF- κ B, as a transcriptional factor controlling a variety

of target genes such as adhesion molecular and apoptosis, is closely related to the pathogenesis and progression of tumors. NF- κ B activity in cells like leukocyte, could be increased by hydrogen peroxide and NF- κ B activation was an essential step before VEGF expression level was increased by hydrogen peroxide in murine osteoblastic cells^[60,61].It is noteworthy in the present experiment that the increase of NF- κ B activity was accompanied by the promotion of expression of VEGF in colon cancer cells exposed to hydrogen peroxide. Thereby, we estimate that the NF- κ B activation may be the prerequisite of the effect of hydrogen peroxide on VEGF expression in colon cancer cells.

In view that such reactive oxygen species as hydrogen peroxide are likely to promote the development of colon cancer, it would be helpful in releasing oxidative stress by antioxidants in the colon cancer therapy.

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