Effect of safrole oxide on vascular endothelial cell growth and apoptosis induced by deprivation of fibroblast growth factor[1]

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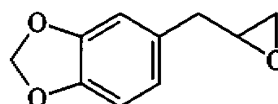
KEY WORDS apoptosis; umbilical veins; vascular endothelium; cell survival; DNA fragmentation; fibroblast growth factor

ABSTRACT

AIM: To investigate effect of safrole oxide on cell growth and apoptosis induced by deprivation of survival factors (fibroblast growth factors, aFGF and bFGF) in vascular endothelial cells (VEC). **METHODS**: Morphological changes were observed by light microscopy. Cell growth was determined by MTT (3-[4, 5-dimethyl thiazol-2-yl]-2, 5-diphenyltetrazolium) method. DNA fragmentation was analyzed by agarose gel electrophoresis and fluorescence microscopy. Cell cycle distribution was analyzed by flow cytometry (FCM). **RESULTS**: The cells deprived of FGF were exposed to safrole oxide 5 - 25 mg/L for 24 h. Cells spreading and growth were promoted ($P < 0.01$), detachment and DNA fragmentation of these cells were suppressed ($P < 0.01$), safrole oxide 10 mg/L had no obvious effect on cell cycle distribution ($P > 0.05$). When the cells were treated with safrole oxide 50 - 100 mg/L, detachment and DNA fragmentation of VEC were promoted ($P < 0.01$). The cell cycle was blocked at G_2-M phase by safrole oxide 100 mg/L. **CONCLUSION**: Safrole oxide 10 mg/L inhibited, but 100 mg/L promoted apoptosis of VEC. Safrole oxide might be an important compound that affects VEC growth and apoptosis.

INTRODUCTION

Safrole oxide has been known for a long time[1], but its pharmacological role is not clear. Recently, the experiment results of Qato and Guenthner show that safrole oxide can bind to calf thymus DNA *in vitro*. However, no corresponding adducts are formed with liver DNA when whole animals are exposed to safrole oxide. They also demonstrated that adducts of safrole oxide and DNA were formed *in vitro* between safrole oxide and deoxyguanosine[2]. Another report also showed that safrole oxide can form covalent adducts with DNA *in vitro*, binding primarily to guanine, but also to the other three DNA bases. Epoxide hydrolases can prevent the binding of safrole oxide to DNA *in vitro*[3]. To date, there has been no report about the effect of safrole oxide on cell growth and apoptosis. Vascular endothelial cells (VEC) play important roles in angiogenesis that is critical for normal physiological processes such as embryonic development and wound repair and also promote tumor growth[4-6]. Therefore, the regulation of apoptosis of VEC is important in wound repair and cancer therapy. In this study, safrole oxide was synthesized, and its effect on growth and apoptosis of VEC was examined to provide experimental evidence for its clinical trial.



Safrole oxide

MATERIALS AND METHODS

Reagents M199 medium was purchased from Gibco BRL Co, Grand Island, NY. Fetal bovine serum (FBS) was obtained from Hycolon Lab Inc USA. Fibroblast growth factor (FGF) was extracted from bovine brains in our laboratory[7]. Safrole [98 % (GC)] was purchased from Fluka AG and 3-chloropero-

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xybenzoic acid from ACROS. Safrole oxide [3, 4-(methylenedioxy)-1-(2, 3-epoxypropyl)-benzene] was synthesized by the reaction of safrole with 3-chloroperoxybenzoic acid, and purified by silica gel column chromatography. All other reagents were of analytical grade grade.

Cell cultures Human umbilical vein endothelial cells (HUVEC) were obtained in our laboratory by the method of Jaffe *et al*[8]. The cells were cultured on gelatin-coated plastic dishes in M199, supplemented with 10 % FBS, and FGF 70 μg/L (as well as heparin 100 mg/L) at 37 ℃ in 5 % CO_2 + 95 % air (normal group). All experiments were performed on cells from 5 – 12 passages.

Cell growth assay When cultured cells reached confluence, the cells were washed once with the medium and replaced with M199 medium in the presence of serum (control group). The cells were incubated with or without safrole oxide for 24 h, cell growth was determined by MTT-assay[9].

Analysis of DNA fragmentation Cells were incubated in a digestion buffer that contained proteinase K 0.2 g/L at 50 ℃ overnight. The cellular DNA was extracted once with phenol and once with a mixture of phenol, chloroform, and 3-methyl-1-butanol (25:24:1, v:v:v). After digested by RNase (final concentration 0.6 g/L) at 37 ℃ for 30 min, the samples were subjected to electrophoresis on a 2 % agarose gel in Tris-acetate buffer. The gel was then stained with ethidium bromide and photographed on an UV transilluminator.

Nuclear fragmentation assay Cells after treatment were washed once with PBS (phosphate-buffered saline), fixed with 1 % glutaraldehyde solution at 25 ℃ overnight, centrifuged (9000 × *g*, 5 min) and resuspended in PBS, and then stained with Hoechst 33258 (Sigma Co) at the concentration of 1 mol/L for 20 min. After three washes with PBS, the cells were mounted onto slides for analysis under a fluorescence microscope.

Determination of cell cycle distribution The cells were cultured in the medium without or with safrole oxide 10 mg/L or 100 mg/L for 12 h. Cell cycle distribution and apoptosis rate directly determined by flow cytometry (Facscan, Bo Co, USA).

Statistical analysis Data were expressed as $\bar{x} \pm s$ and analýzed by *t*-test.

RESULTS

Cell growth and morphological changes When the cells were deprived of FGF from M199 medium with serum, cell growth was inhibited ($P < 0.01$) (Tab 1), the cells gradually detached from the dish and apoptosis occurred (Fig 1). After exposure of VEC to safrole oxide 5 – 25 mg/L for 24 h, VEC growth was promoted ($P < 0.01$) (Tab 1), cell detachment was inhibited, and some of the cells elongated and spreaded over the dish (Fig 1). When the cells were treated with safrole oxide 50 – 100 mg/L for 24 h, cell growth was obviously suppressed ($P < 0.01$) (Tab 1) and almost all of the cells floated in the medium, many apoptotic bodies appeared (Fig 1).

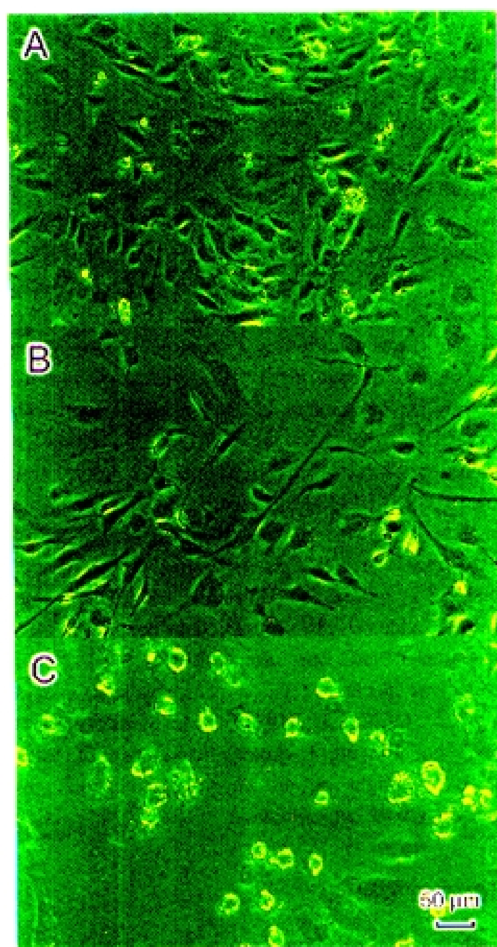

Fig 1. Morphological changes of VEC at 24 h. A) Control; B) Cells treated with safrole oxide 10 mg/L; C) Cells treated with safrole oxide 100 mg/L. × 200.

DNA fragmentation Electrophoresis of DNA extracted from the cells deprived of FGF in the presence

Tab 1. Effect of safrole oxide on cell growth of VEC at 24 h. $n = 5$. $\bar{x} \pm s$. bP<0.05, cP<0.01 vs control group.

Drug/mg·L^{-1}	A_{570}	Viability/%
Normal	0.377±0.008c	100
Control	0.15±0.05	39.4±1.9
Safrole oxide 5	0.168±0.017b	44.7±0.8b
10	0.36±0.04c	94.7±1.8c
25	0.239±0.028c	63.1±1.0c
50	0.083±0.012c	21.1±0.4c
75	0.041±0.023c	10.5±0.8c
100	0.023±0.008c	5.2±0.4c

of serum for 24 h showed a "ladder" pattern. DNA ladders from the cells treated with safrole oxide 10 mg/L were less intense than that from untreated cells, but safrole oxide 100 mg/L promoted the DNA fragmentation (Fig 2).

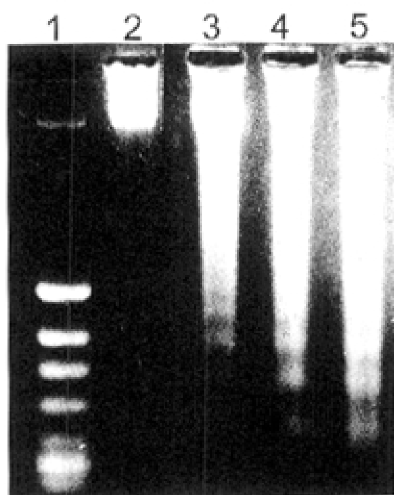

Fig 2. Effect of safrole oxide on DNA fragmentation in apoptotic VEC deprived of FGF at 24 h. 1) DNA marker (fragments of φx-174 DNA, 79 – 1057 bp); 2) DNA from normal cells; 3) DNA from cells treated with safrole oxide 10 mg/L; 4) DNA from control cells; 5) DNA from cells treated with safrole oxide 100 mg/L.

Nuclear fragmentation When the cells were exposed to safrole oxide 10 mg/L for 24 h, the nuclear fragmentation induced by deprivation of FGF was suppressed, while the cells were treated with the drug 100 mg/L, the nuclear fragmentation was promoted (Fig 3).

Cell cycle distribution and apoptosis rate After VEC was exposed to safrole oxide 10 mg/L for

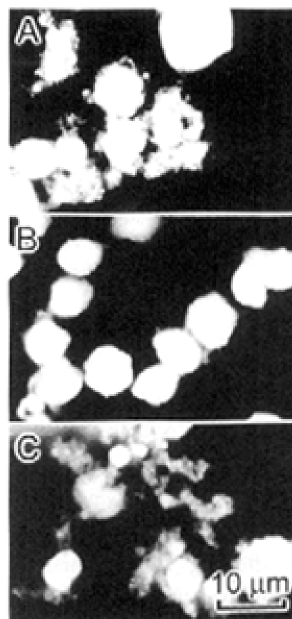

Fig 3. Nuclear fragmentation of VEC stained with Hoechst 33258 at 24 h. A) Control; B) The nuclei of cells treated with safrole oxide 10 mg/L; C) The nuclei of cells treated with safrole oxide 100 mg/L. ×1000.

12 h, there were no obvious changes of cell cycle distribution and apoptosis rate. When the dose was 100 mg/L, the percentage of cells in G_0-G_1 and S phase decreased ($P<0.01$), and the number of cells in G_2-M phase and apoptosis cells increased ($P<0.01$) (Tab 2).

DISCUSSION

Safrole oxide has piperonyl and epoxy structures which are important in many compounds with phisological activity[10], but its effect on cell growth and apoptosis of VEC is not known. We have been studying VEC growth and apoptosis induced by deprivation of FGF as a model of research on angiogenesis and blood vessel degeneration *in vitro*[11,12]. In present study we used this model in order to find a regulator of VEC growth and apoptosis. The data showed that at low concentration (5 – 25 mg/L) safrole oxide promoted cell growth and cell spreading, and inhibited the DNA fragmentation induced by deprivation of FGF, and that at high concentration (50 – 100 mg/L) the drug blocked cell cycle at G_2-M phase and promoted VEC apoptosis. Our results provided strong evidence for the first time that safrole oxide might be a regulator of VEC growth and apoptosis.

Normal endothelial and epithelial cells denied

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Tab 2. Effect of safrole oxide on cell cycle distribution in VEC at 12 h. $n=3$. $\bar{x} \pm s$. [a]$P>0.05$, [c]$P<0.01$ *vs* control group.

Drug/mg·L^{-1}	G_0-G_1/%	G_2-M/%	S/%	Apoptosis/%
Normal	60.2±1.2	16.4±1.0	23.3±1.2	2.8±0.3
Control	61.5±1.4	21.9±1.5	16.6±0.9	3.24±0.23
Safrole oxide 10	58.5±1.0[a]	23.3±1.2[a]	18.2±0.8[a]	3.2±0.4[a]
100	47.2±1.5[c]	51.8±1.8[c]	1.0±0.5[c]	20.2±1.0[c]

anchorage undergo apoptosis, which is called anchorage-dependent phenomenon. The ability of anchorage-dependent cells to proliferate is linked to spreading. In human vascular endothelial cells the extent to which cells spread is related to their viability, when the cells become round in shape they stop growing[13]. The results demonstrated that safrole oxide might regulate cell growth and apoptosis by affecting VEC anchorage which is very important in cell survival signal transduction[14].

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黄樟素氧化物对去除成纤维细胞生长因子所诱导的血管内皮细胞凋亡及生长的影响[1]

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关键词 细胞凋亡; 脐静脉; 血管内皮; 细胞存活; DNA 断片; 成纤维细胞生长因子

目的: 研究黄樟素氧化物对去除成纤维细胞生长因子(FGF)诱导的血管内皮细胞凋亡及生长的影响. **方法**: 光学显微镜观察细胞形态学变化; MTT 法测定细胞生长; DNA 电泳和荧光显微技术检测 DNA 断裂; 流式细胞术测定细胞周期分布. **结果**: 黄樟素氧化物 5-25 mg/L 处理去除 FGF 的血管内皮细胞 24 h, 细胞铺展和生长被促进, 细胞脱壁和 DNA 片段化被抑制, 黄樟素氧化物 10 mg/L 对细胞周期分布无明显影响. 黄樟素氧化物 50-100 mg/L 促进血管内皮细胞的脱壁和 DNA 片段化, 黄樟素氧化物 100 mg/L 将细胞周期阻断于 G_2-M 期. **结论**: 黄樟素氧化物在 10 mg/L 时抑制血管内皮细胞凋亡, 而在 100 mg/L 时促进其凋亡, 该药物对血管内皮细胞生长和凋亡有重要影响.

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