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Effect of manoalide on apoptosis induced by deprivation of growth factors in vascular endothelial cells

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KEY WORDS manoalide; apoptosis; umbilical veins; vascular endothelium; cultured cells; cell survival; DNA fragmentation; acidic fibroblast growth factor

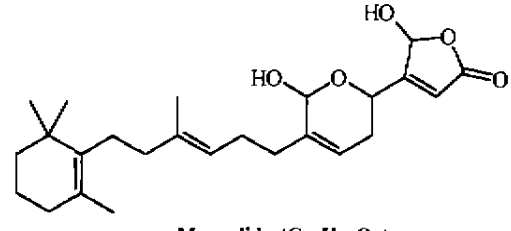
ABSTRACT

AIM: To study effect of manoalide on apoptosis induced by deprivation of acidic fibroblast growth factor (aFGF) and serum in vascular endothelial cells (VEC). **METHODS**: Morphologic changes were observed by light microscopy. Viability was determined by counting the cells that attached to dishes after treatments. DNA fragmentation was analyzed by agarose gel electrophoresis and fluorescence microscopy. **RESULTS**: The cells deprived of aFGF and serum were exposed to manoalide 1 - 4 $\mu mol \cdot L^{-1}$ for 48 h, detachment and DNA fragmentation of these cells were suppressed. At 7 $\mu mol \cdot L^{-1}$, manoalide promoted detachment and DNA fragmentation of VEC. **CONCLUSION**: manoalide 2 $\mu mol \cdot L^{-1}$ inhibited, but 7 $\mu mol \cdot L^{-1}$ promoted, apoptosis of VEC.

INTRODUCTION

Endothelial cells play important roles in the formation of blood vessels (angiogenesis or neovascularization) and their degeneration[1]. Angiogenesis is critical for normal physiologic processes such as embryonic development and wound repair[2]. However, angiogenesis also facilitates tumor growth, retinopathy, and various inflammatory disorders[3]. Therefore, the importance of inhibiting and inducing apoptosis of vascular endothelial cells (VEC) in wound repair and cancer therapy is commonly accepted[4], and it is desirable to look for new drugs that induce or inhibit apoptosis of VEC.

Manoalide is a sesterterpenoid soluble in methanol, ethanol, and Me_2SO. It is isolated from the marine sponge *Luffarinella variabilis*, and owns potent anti-inflammatory activity[5]. In our previous paper, we found that manoalide induced apoptosis of VEC at 7 $\mu mol \cdot L^{-1}$ as an inhibitor of phospholipase A_2[6]. In this paper, we studied the effect of manoalide on apoptosis of VEC at lower concentration comparing with that at higher concentration, so as to clarify the dose-response relationship between manoalide and VEC apoptosis and to provide experimental evidence for its clinical trial.



Manoalide ($C_{25}H_{36}O_5$)

MATERIALS AND METHODS

Reagents MCDB-104 medium was purchased from Kyokuto Pharmaceutical Industries, Tokyo, Japan. Fetal bovine serum (FBS) was

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purchased from Wako Industries, Tokyo. Acidic fibroblast growth factor (aFGF) was extracted from bovine brains[7]. Manoalide was purchased from Wako Industries. All other reagents were of ultrapure grade.

Cell cultures Human umbilical vein endothelial cells (HUVEC) were obtained[8]. The cells were cultured on gelatin-coated plastic dishes in MCDB-104, supplemented with 10 % FBS, and aFGF 70 $\mu g \cdot L^{-1}$ (as well as heparin 100 $mg \cdot L^{-1}$) at 37 ℃ in 5 % CO_2 + 95 % air. Cells with a population doubling level of 15 to 25 were used.

Viability assay When cultured cells reached confluence, the cells were washed once with the medium and replaced with MCDB-104 medium. The cells were incubated with or without manoalide. Trypsinized cells were counted with a Coulter counter after 12, 24, 36, and 48 h. Detached cells were washed away before the treatment with trypsin. The cells that remained attached to dishes after washing away of blebs were not stained by erythrosin B ($5 g \cdot L^{-1}$, Sigma) and were regarded as living cells.

Analysis of DNA fragmentation Cells were incubated in a digestion buffer that contained proteinase K $0.2 g \cdot L^{-1}$ 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, vol/vol). After digestion by RNase (final concentration $0.6 g \cdot L^{-1}$) 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 a 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 and resuspended in PBS, and then stained with Hoechst 33258 at the concentration of $1 mol \cdot L^{-1}$ for 20 min. After three washes with PBS, the cells were mounted onto slides for analysis under a fluorescence microscope.

Statistics Data were expressed as $\bar{x} \pm s$ and analyzed by t test.

RESULTS

Effect of manoalide 1 – 4 $\mu mol \cdot L^{-1}$ on VEC apoptosis After deprivation of aFGF and serum, cells gradually started to round up and eventually became detached from the dish and floated in the medium. The plasma membrane of rounded-up cells developed blebs and apoptotic bodies formed from these cells. When cultures that had been depleted of growth factor were treated with manoalide 1 – 4 $\mu mol \cdot L^{-1}$, no significant differences in cell morphology between manoalide-treated and untreated cells were observed up to 6 h. From then, some of the cells gradually started to elongate and spread over the dish. These cells remained attached to the dish while other cells without such morphologic changes seemed to round up continuously and became detached from the dish (Fig 1).

Consequently, the number of cells (40 % – 66 %) attached to the dish in manoalide-treated cultures was higher than that (30 %) in untreated cultures. The maximal effect of manoalide was observed at a concentration of 2 $\mu mol \cdot L^{-1}$ ($P < 0.01$) (Tab 1).

Tab 1. Effect of manoalide on apoptosis of VEC. The numbers of cells that remained on dishes were counted 48 h after deprivation of aFGF and serum and treatment with manoalide. $n = 5$ experiments. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Manoalide/$\mu mol \cdot L^{-1}$	Viability/%
0	30.2 ± 1.1
1	45.3 ± 2.4^b
2	65.8 ± 3.6^c
3	51.3 ± 3.2^c
4	39.7 ± 2.8^b

Fig 1. VEC cultured in MCDB medium with (A) or without (B) aFGF and serum for 36 h. (C) Treatment with manoalide 2 μmol·L^{-1} for 36 h (×100)

The number of surviving cells in the presence of manoalide 2 μmol·L^{-1} was about twice that in the absence of the reagent after a 48-h incubation ($P<0.01$) (Tab 2).

Tab 2. Inhibition of apoptosis in VEC by manoalide 2 μmol·L^{-1}. The cells that remained on dishes were counted 12, 24, 36, and 48 h after the start of treatment. $n=5$ experiments. $\bar{x}\pm s$. $^aP>0.05$, $^cP<0.01$ *vs* control.

Time/h	Viability/% Control	Manoalide-treated
12	85.2±3.3	89.6±3.8[a]
24	60.4±2.1	80.2±3.1[c]
36	45.3±3.8	71.4±2.8[c]
48	30.4±1.9	62.3±3.1[c]

DNA from whole culture dish was isolated and analyzed at 24 h after application of the apoptotic stimulus with or without manoalide. The rungs on DNA ladders from treated cells were obviously less intense than those from untreated cells. The amount of DNA with large molecular mass was much greater in treated cells than that in untreated cells (Fig 2).

Fig 2. Effect of manoalide 2 μmol·L^{-1} on DNA fragmentation in apoptotic VEC deprived of aFGF and serum. (1) Deprivation of aFGF and serum. (2) Deprivation of aFGF and serum, and addition of manoalide. (3) DNA markers.

Effect of manoalide 5 – 10 μmol·L^{-1} on VEC apoptosis When VEC were exposed to manoalide 5 – 10 μmol·L^{-1} in the absence of aFGF and serum, the cells died more rapidly than control cells, and almost all of the cells disintegrated into apoptotic bodies within 16 h at 7 μmol·L^{-1} (Fig 3B). After 16 h of such treatment the cells were collected by centrifugation and were stained with Hoechst 33258 (Fig 3D).

At the same time, we extracted and analyzed the DNA of manoalide-treated cells. Promotion of nuclear fragmentation by manoalide confirmed that the death of VEC was apoptotic cell death (Fig 4).

DISCUSSION

The results suggested that when the

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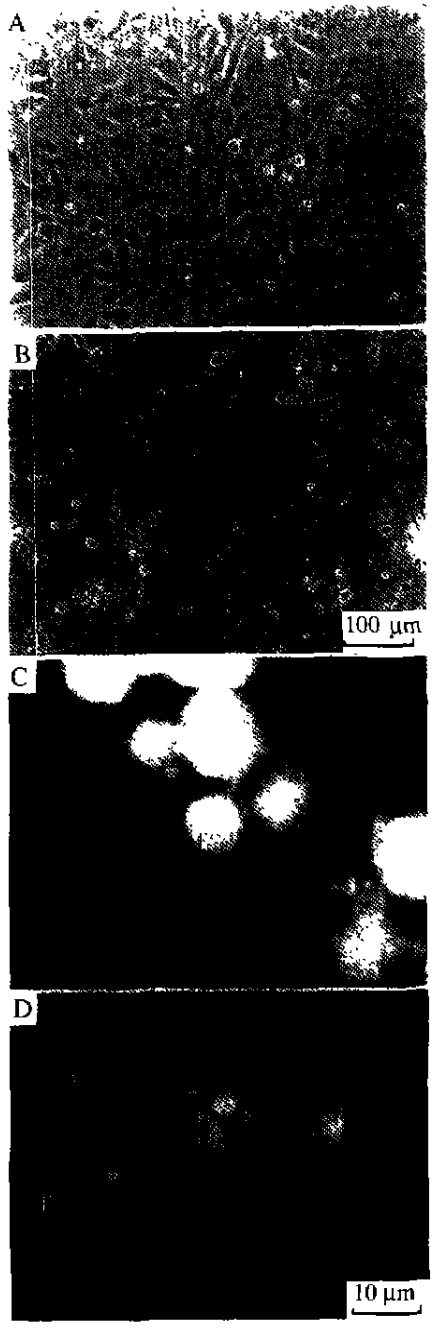

Fig 3. Promotion of apoptosis in VEC by manoalide 7 μmol · L^{-1}. Morphologic changes and fluorescence of VEC after incubation for 16 h. A and C: Deprivation of aFGF and serum. B and D: Deprivation of aFGF and serum, and addition of manoalide. (A and B: × 100; C and D: × 1000).

concentration of manoalide was in range of 1 – 4 μmol · L^{-1}, the drug inhibited apoptosis of VEC

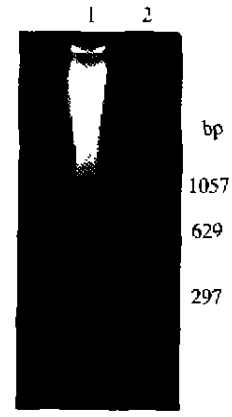

Fig 4. Effect of manoalide 7 μmol · L^{-1} on DNA fragmentation in apoptotic VEC deprived of aFGF and serum. (1) Treatment with manoalide for 16 h; (2) DNA markers.

induced by deprivation of aFGF and serum. The maximal effect of manoalide was observed at a concentration of 2 μmol · L^{-1}. The evidence of morphology and nuclear fragmentation presented here demonstrated that at higher concentrations of manoalide (5 – 10 μmol · L^{-1}), it promoted apoptosis of VEC (data not shown). The most effective concentration was 7 μmol · L^{-1}[6]. This dose-response effect of manoalide on apoptosis is common in cell biology, such as that of cycloheximide and hydrogen peroxide[9,10]. Our result provided another example.

Manoalide has been shown to act on phospholipases, whose activities are indispensable for the regulation of lipid metabolism in membranes, as well as for the maintenance of membrane stability[11]. Manoalide 7 μmol · L^{-1} inhibited phospholipase A_2 and promoted apoptosis of VEC[6], but the mechanism by which manoalide 2 μmol · L^{-1} inhibited apoptosis of the cells is not clear. The evidence presented in this study indicated that manoalide had more than one target molecule in its action on apoptosis of VEC. Identification of the target molecules in the cells is obviously necessary for understanding the action of manoalide.

Human endothelial cells become flatter and they stop growing as they become increasingly round in shape. It seems likely, therefore, that the extent to which cells spread is related to their viability[12,13]. Our data demonstrated that manoalide might affect the anchoring of VEC, such that the cells became flatter and spread or round in shape. This phenomenon is most probably a reflection of the way in which manoalide affects the process of apoptosis. Our data provided strong evidence for the first time that manoalide at 2 $\mu mol \cdot L^{-1}$ inhibited VEC apoptosis and gave more information on the therapeutic potential of the drug.

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Manoalide 对除去生长因子诱导的血管内皮细胞凋亡的影响

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关键词 manoalide；凋亡；脐静脉；血管内皮；培养的细胞；细胞存活；DNA 断片；酸性成纤维细胞生长因子

目的：研究 manoalide 对除去生长因子(aFGF 和血清)而诱导的血管内皮细胞凋亡的影响. **方法**：通过细胞形态观察，DNA 凝胶电泳及荧光显微术等方法确定 manoalide 对细胞凋亡的抑制或促进作用. **结果**：向去除 aFGF 和血清的培养液中加低浓度的 manoalide (1 - 4 $\mu mol \cdot L^{-1}$). 培养细胞 48 h，细胞的脱壁和 DNA 片断化受到抑制；manoalide 浓度为 7 $\mu mol \cdot L^{-1}$时，促进细胞脱壁和 DNA 片断化. **结论**：低浓度的 manoalide (2 $\mu mol \cdot L^{-1}$)抑制血管内皮细胞凋亡，而较高浓度的 manoalide (7 $\mu mol \cdot L^{-1}$)促进该细胞凋亡.

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