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Original article

Direct effects of fascaplysin on human umbilical vein endothelial cells attributing the anti-angiogenesis activity

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ABSTRACT

Novel anti-angiogenesis activity of fascaplysin via VEGF blockage was recently revealed by our previous study in addition to the reported cyclin-dependent kinase 4 (CDK4) selective inhibition. To uncover more details of this pharmacologically prospective property, this study further investigated whether fascaplysin had direct anti-proliferation effects on human umbilical vein endothelial cells (HUVEC), which might be contributing to anti-angiogenesis. The results showed that G1 cell cycle arrest was induced by 2.6 μM fascaplysin in a time-dependent manner, and exhibited more sensitive than hepatocarcinoma cells BeL-7402 and Hela cells. Approximately 56.09 ± 2.63% of the cells were arrested at the G1 phase after 24 h, and 64.94 ± 2.07% after 36 h, comparing to the 22.82 ± 1.2% in methanol treated cells. Apoptosis of HUVEC cells was induced by 1.3 μM fascaplysin and indicated by the sub-G1, Hoechst staining, terminal deoxynucleotidyl transferase dUTP-mediated nicked end labeling (TUNEL) assay, and annexin-V and propidium (PI) label. This apoptosis response was further confirmed by the detection of active caspase-3 and by western blotting using antibodies against Bax, Bcl-2, procaspase-8, and Bid, indicating that apoptosis in HUVEC cells may involve a mitochondria pathway, by the demonstration of an increase in the Bax/Bcl-2 ratio. Together, our results suggest that the anti-angiogenesis activity of fascaplysin is through the direct effects of cell cycle arrest and apoptosis on HUVEC.

1. Introduction

The hypothesis that "tumor growth is angiogenesis dependent" was first proposed by Folkman in 1971 and is used today to conceive better cancer therapy. Angiogenesis, the formation of new blood vessels from preexisting vessels, has been shown to be essential for tumor growth and metastasis. Given to the molecular and cellular mechanisms of angiogenesis, the strategies used to develop anti-angiogenic agents are mainly two, interfering with signaling pathways of angiogenesis, and directly targeting tumor vasculature [1]. Generally speaking, the majority of the anti-angiogenic agents are targeting the signaling pathway of angiogenesis, especially the VEGF pathway [2–5]. On the other hand, targeting the tumor vasculature is achieved through inhibition of endothelial cell proliferation or activation of endothelial cell apoptosis. In this way, the source of new blood vessels are destroyed, which may prevent further tumor growth, and tumor cells are starved leading to cell death directly. As endothelial cells are genetically stable and exhibit lower mutagenesis rate than tumor cells, the possibility of drug resistance will be reduced [6]. Targeting the tumor vasculature is getting more and more attention of reaserchers, and several endothelial cell models have been developed in relative researches. According to the literatures, the most widely used endothelial cell model is human umbilical vein endothelial cell (HUVEC) [7–9]. Taken together, tumor viability is dependent on the nutrients provided by the vasculature, and tumor growth is dependent on the new blood vessel formation. Therefore, in theory, a promising anti-angiogenic agent for cancer therapy should not only block the new blood vessel formation, but also kill or destroy the tumor vasculature.

In our previous study, we have demonstrated fascaplysin (Fig. 1), which was originally isolated from Fijian marine sponge *Fascaplysinopsis* sp. in 1988 [10], is an interesting angiogenesis inhibitor [11]. It inhibited capillary plexus formation and suppressed VEGF expression in the chorioallantoic membrane model. Moreover, it inhibited VEGF expression and secretion by human hepatocarcinoma cells BeL-7402, and shown selective inhibition of HUVEC cells towards BeL-7402 cells by the MTT assay.

It is known that fascaplysin is a significant cyclin-dependent kinase 4 (CDK4) inhibitor. Study has demonstrated that fascaplysin-arrested osteosarcoma cells U2OS, colon carcinoma cells HCT116 and diploid fibroblasts cells MRC-5 in G1 cell cycle [12].

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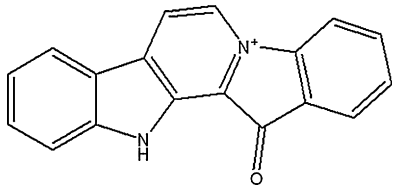

Fig. 1. Chemical structure of fascaplysin.

Therefore, we would like to obtain more details of fascaplysin's direct anti-proliferation effects on human umbilical vein endothelial cells, which might attribute to the angiogenesis activity besides blocking the VEGF excretion. In the current study, we have shown that fascaplyisn inhibited human umbilical vein endothelial cell proliferation and activated human umbilical vein endothelial cell apoptosis.

2. Materials and methods

2.1. Reagents

Minimum essential medium (MEM) was purchased from Gibco invitrogen corporation (Australia), and fetal bovine serum (FBS) was purchased from PAA Laboratories GmbH (Austria). The CycleTest™ plus DNA reagent kit for cell cycle analysis was purchased from Becton Dickinson (China). The FragEL™ DNA fragmentation detection kit for TUNEL assay was purchased from Merk (China). The annexin V-FITC apoptosis detection kit was purchased from Becton Dickinson (China). The FITC-conjugated monoclonal active caspase-3 antibody apoptosis kit I was purchased from BD Biosciences (China). Bio-Rad Dc protein assay was purchased from Bio-Rad (China). Antibodies against Bax, Bcl-2, Bid and procaspase-8 were purchased from Santa Cruz (China). All other reagents were of highest analytical grade.

2.2. Cell lines and cell culture

Human umbilical vein endothelial cells (HUVEC), human hepatocarcinoma cells (BeL-7402) and human cervical carcinoma cells (Hela) were obtained from China Center for Type Culture Collection (Wuhan, China). Cells were maintained in MEM supplemented with 10% FBS, 100 U ml-1 penicillin and streptomycin at 37 °C in a humidified atmosphere of 5% CO_2, and subcultured upon reaching 80% confluence.

2.3. Cell cycle analysis

After fascaplysin treatment, cells were trypsinized, centrifuged at 1500 rmp for 10 min, washed thrice with cold PBS. The cells were pelleted and analyzed using CycleTest™ plus DNA reagent kit (Becton Dickinson, China), according to the manufacturer's instructions. The pellets were washed thrice with Buffer Solution, resuspended in Solution A and incubated at room temperature for 10 min. Solution C was adding thereafter and cells were incubated at 4 °C in dark for 10 min following the incubation with additional Solution B for 10 min. Cell cycle analysis was performed using flow cytometry (BD Bioscience, USA), and percentages of cells in G1, S, and G2 phase were calculated using Modfit LT 3.0 program (San Jose, CA).

2.4. Morphological observation of apoptotic cells

To determine the cell morphologic changes, Hoechst staining was carried out. Cells were washed with phosphate-buffered saline (PBS), fixed with methanol/acetic acid (3:1) for 15 min, and then incubated with 1% Hoechst for 45 min. Observation was performed under a fluorescent microscope (BD Bioscience, USA).

2.5. TUNEL staining

To identify apoptotic cells, TUNEL reaction was performed according to the manufacturer's instructions (Merk, China). Briefly, cells were trypsinized thereafter, centrifuged at 1500 rmp for 10 min, washed thrice with cold PBS. The cells were fixed with 4% paraformaldehyde for 60 min at room temperature, washed thrice with PBS, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate and then rinsed with PBS. Cells were stained with 50ul TUNEL reaction mixture at 37 °C for 60 min, washed with PBS. Afterwards, the cells were viewed using a fluorescence microscopy.

2.6. Detection of apoptosis

Apoptosis rates were measured by using an annexin V-FITC apoptosis detection kit (BD Bioscience, China). Cells were harvested, washed twice with PBS, resuspended in 1 × binding buffer. The cells were incubated with 5 μl annexin V-FITC and 5 μl PI at 25 °C for 15 min in dark. Apoptosis was analyzed by flow cytometry.

2.7. Active caspase-3 assay

The HUVEC cells were treated with 1.3 μM of fascaplysin for various periods of time, and then incubated with FITC-conjugatedd monoclonal rabbit anti-activie-caspase-3 according to the manufacturer's instructions (BD Biosciences, China). Briefly, cells were washed twice with PBS, incubated in Cytofix/Cytoperm™ solution for 20 min on ice to be fixed and permeabilized. The cells were pelleted by centrifugation, washed with Perm/Wash™ buffer, and then incubated with antibody for 30 min at room temperature. After being washed with Perm/Wash™ buffer, cells were analyzed using flow cytometry.

2.8. Western blotting analysis

After 1.3 μM of fascaplysin treatment for 3, 6, 12, 24 h, protein extracts were prepared for western blotting analysis. The soluble protein concentration was determined using Bio-Rad Dc protein assay (Bio-Rad, China). Proteins were subjected to 12% SDS-PAGE gel, transferred onto a polyvinylidene difluoride (PVDF) membrane, and incubated overnight at 4 °C with a primary antibody at appropriate dilution before incubation with a secondary antibody conjugated to horseradish peroxidase (HRP) at a dilution of 1:2000 for 1 h at room temperature. The protein was viewed using chemiluminescence solution from Beyotime (China). β-actin was used as an internal control.

3. Results

3.1. G1 cell cycle arrest

In order to better depict the control of cell cycle distribution by fascaplysin, HUVEC cells were treated with 1.3 μM (EC50), 2.6 μM fascaplysin for 24 h, 36 h, respectively. There was a significant decrease in the population of cells in G2 and a significant increase in the popuplation of cells in G1 phase associated with the present of 2.6 μ M fascaplysin, in a time-dependent manner (Fig. 2). Approximately 56.09 ± 2.63% of the cells were arrested at the G1 phase after 24 h, and 64.94 ± 2.07% after 36 h, comparing to the 22.82 ± 1.2% in control (methanol treated) cells. By comparing the data to that of BeL-7402 and Hela cells, HUVEC cells performed more

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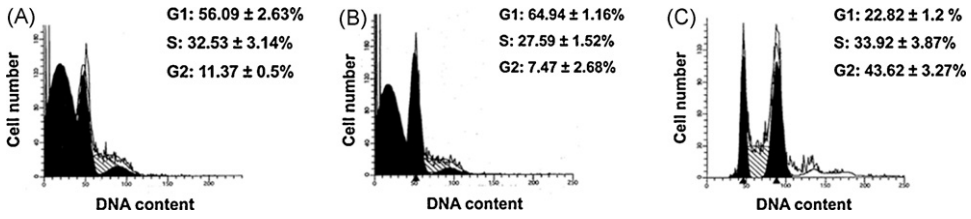

Fig. 2. Fascaplysin induced G1 cell cycle arrest in HUVEC cells. Cells were treated with 2.6×10^{-6} M fascaplysin for 24 h, 36 h, and cell cycle distribution was detected by flow cytometry. Percentages of cells in each phase of the cell cycle were calculated using Modfit LT 3.0 program. Histograms show one representative experiment. Values are means and SD calculated from three independent replicates. **A.** Cells were treated by 2.6×10^{-6} M fascaplysin for 24 h. **B.** Cells were treated by 2.6×10^{-6} M fascaplysin for 36 h. **C.** Cells were treated by methanol as control.

Table 1
Cell cycle distribution of fascaplysin-treated BeL-7402 cells.

	G1 (%)	S (%)	G2 (%)
Control	67.04 ± 0.38	22.8 ± 1.28	10.16 ± 0.9
2 μM, 24 h	71.55 ± 1.78*	21.1 ± 2.25	7.34 ± 0.48**
2 μM, 36 h	61.11 ± 1.19***	23.47 ± 2.47	15.42 ± 1.28*
4 μM, 24 h	76 ± 1.5***	16.66 ± 0.66*	7.34 ± 0.848*
4 μM, 36 h	49.63 ± 1.46***	40.86 ± 0.05	9.51 ± 1.51

Data is presented as mean values ± SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to control.

Table 2
Cell cycle distribution of fascaplysin-treated Hela cells.

	G1 (%)	S (%)	G2 (%)
Control	70.8 ± 3.13	21.1 ± 0.6	8.1 ± 2.01
0.5 μM, 24 h	63.12 ± 2.68*	25.97 ± 1.87*	10.91 ± 0.81
1.1 μM, 24 h	45.92 ± 0.87***	36.84 ± 2.64***	17.24 ± 1.77**
1.9 μM, 24 h	59.86 ± 0.55	21.67 ± 1.49*	7.62 ± 0.94
2.6 μM, 24 h	64.97 ± 1.87*	12.42 ± 0.81**	6.7 ± 2.64

Data is presented as mean values ± SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to control.

susceptible to the G1 cell cycle arrest activity of fascaplysin than tumor cells. BeL-7402 revealed slight G1 phase arrest within a higher concentration drug treatment (4 μM) (Table 1), and Hela cells failed to arrest at this phases (Table 2) in a series of concentration around the EC50. Additionally, dramatic increase of sub-G1 peaks were observed in the histograms indicating apoptotic cells death (Fig. 2).

3.2. Morphological changes in HUVEC cells

DNA analysis of detecting sub-G1 peak has been adopted widely as one of the reliable biochemical markers of apoptosis [13–15]. As sub-G1 peaks had been observed by the flow cytometry, we determined to examine morphological changes of HUVEC cells induced by fascaplysin to confirm the apoptosis. HUVEC cells were treated with 1.3, 2.6 μM fascaplysin in methanol for 24, 36 h, respectively. Cells treated with methanol were used as control treatment. Hoechst staining showed that HUVEC displayed apoptotic morphology characters in a dose and time-dependent manner. Typical morphologic features of apoptosis, such as condensation of chromatin and nuclear segmentation (shown by the arrows) were observed in all treatments except control treatment. Staining results of HUVEC cells with 1.3 μM fascaplyisn were shown in Fig. 3.

3.3. Apoptotic HUVEC cells detection

Apoptotic HUVEC cells were detected by TUNEL staining. The TUNEL assay showed that few nuclei of fascaplysin-treated cells were staining positive comparing to the control treatment in a dose and time-dependent manner, suggesting that these cells may be undergoing apoptosis with the evidence of the production of DNA strand breaking (Fig. 4).

3.4. Flow cytometric assessment of HUVEC cells apoptosis

The same conditions of fascaplysin treatment were applied to HUVEC cells in flow cytometry assay. Cells were stained with annexin V-FITC and PI, and subsequently assessed by flow cytometry. As shown in Fig. 5, significant increase of the early stage apoptosis in HUVEC cells was observed at 1.3 μM facaplysin from 1.43 ± 1.41% to 6.27 ± 0.37% after 24 h, to 11.25 ± 2.89% after 36 h. Increasing the drug concentration to 2.6 μM increase the percentage of the late apoptotic cells from 16.21 ± 2.5% to 68.12 ± 0.32% (24 h), and from 29.52 ± 1.52% to 84.52 ± 3.38% (36 h), whereas the early apoptotic cells reduced to 4.54 ± 0.22% (24 h), 3.08 ± 2.31% (36 h). These results showed that the number of total (early + late) apoptotic cells was increased by fascaplysin in time- and dose-dependent manner.

3.5. Fascaplysin induced activation of caspase-3

Caspase-3 is one of the key executioners of apoptosis, and its activation is a good marker for apoptosis [16–18]. The level of active caspase-3 affected by fascaplysin was measured. HUVEC

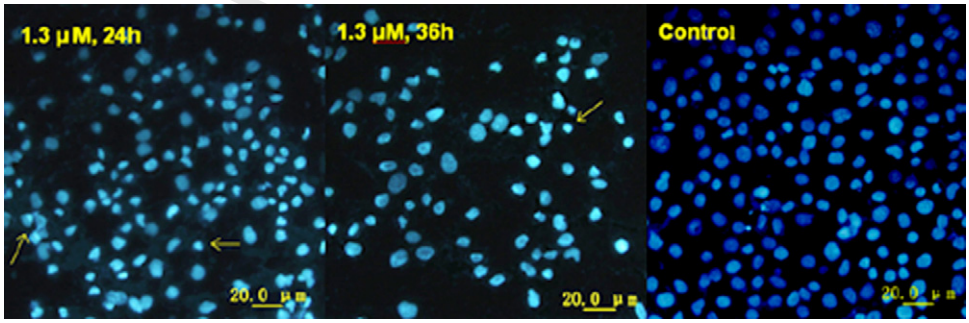

Fig. 3. Hoechst staining of HUVEC cells after exposed to 1.3×10^{-6} M fascaplysin for 24 h, 36 h. Cells treated by methanol were show as control. Arrows show the apoptotic cells.

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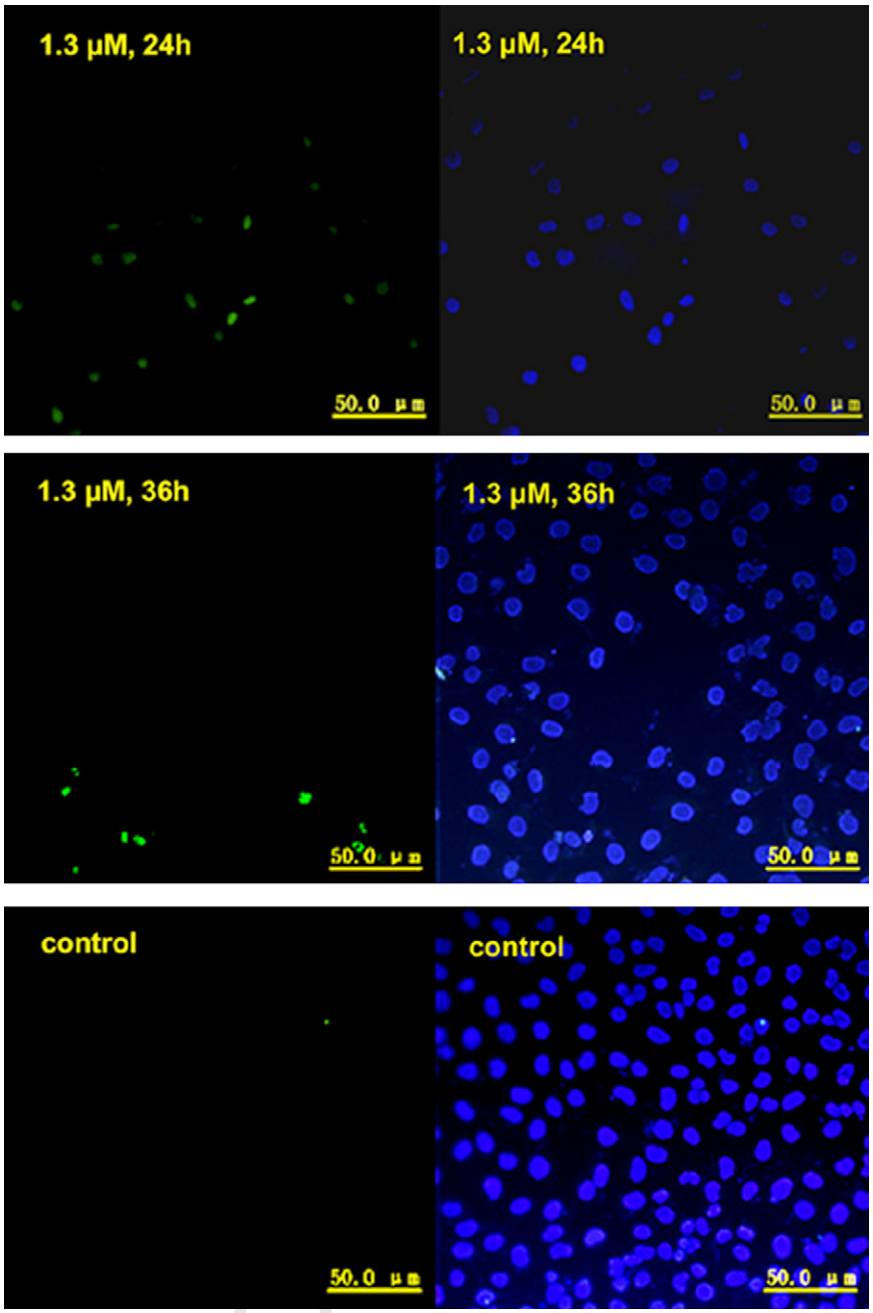

Fig. 4. TUNEL staining. (A, B). Cells were treated with 1.3×10^{-6} M of facaplysin for 24 h. (C, D) Cells were treated with 1.3×10^{-6} M of facaplysin for 36 h (E, F) control.

cells were treated with 1.3 μM facaplysin for several time points and active caspase-3 was assessed by using FITC-conjugated monoclonal active caspase-3 antibody and detected by flow cytometry analysis. The results in Fig. 6 represent percentages of active caspase-3 positive cells (M2) out of total number of counted cells (M1 + M2). The population of positive cells was increased from 3.75% in control to 5.57% at 6 h, 12.26% at 12 h, 18.31% at 24 h, and 22.22% at 36 h, suggesting that caspase-3 pathway was associated with fascaplysin-induced apoptosis in HUVEC cells.

3.6. Fascaplysin altered Bcl-2 family activity and caspase-8 activation

The expression of several key proteins, including anti- and pro-apoptotic proteins, following fascaplysin treatment was examined by western blotting. The time-course analysis showed that at 1.3 μM fascaplysin decreased the expression of Bcl-2 within 3 h of exposure, and that this reduction persisted for 24 h, while the level of Bax remained unchanged but slightly increased after 24 h (Fig. 7). The expression of procaspase-8 was determined by western blotting. As shown in Fig. 7, the protein level of procaspase-8 in HUVEC cells was decreased in a time-dependent manner when exposed to 1.3 μM fascaplysin, which indicated that procascapse-8 might be activated by cleavage. To further confirm the participation of caspase-8 in the apoptosis induction, the level of Bid, which was reported to be activated by caspase-8 mediated cleavage [19,20], was investigated. Result showed that the level of Bid was also decreased by fascaplysin after 3 h, and showed same trend as procaspase-8. These results suggested that the apoptotic effects of fascaplysin in HUVEC cells were associated with an altered protein level of Bcl-2 family and caspase activation.

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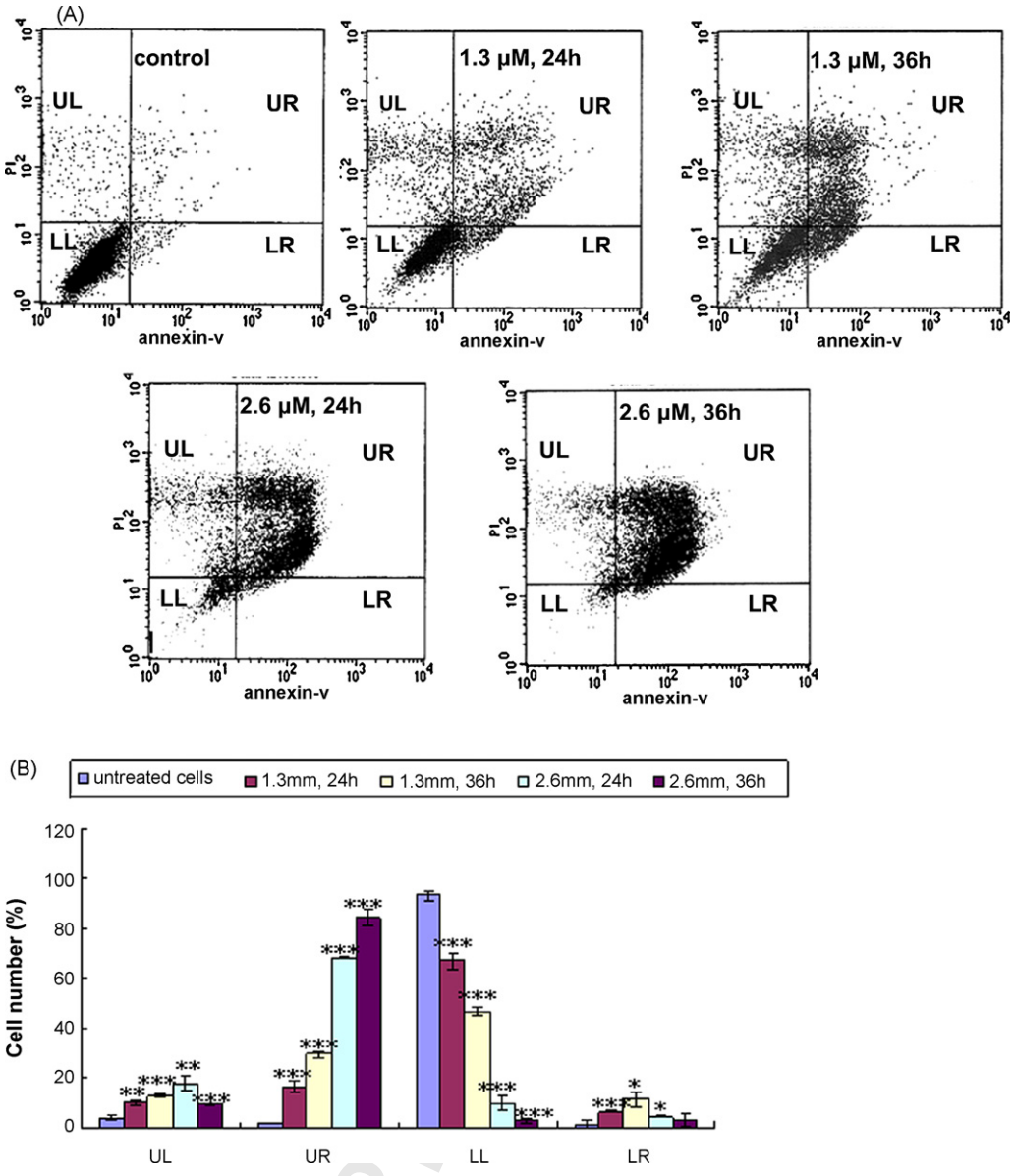

Fig. 5. HUVEC apoptosis determined by annexin V-FITC and PI staining using flow cytometry. $n = 3$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to control. LL: normal cells, LR: the early apoptotic cells, UL: necrotic cells, UR: the lare apoptotic cells.

4. Discussion

Angiogenesis is a complex process that is mediated by the endothelial cells that line blood vessels, and regulated by a number of stimulators such as vascular endothelial growth factor (VEGF) [2,4,21], basic fibroblast growth factor (bFGF) [22,23], and endostatin [24,25]. Therefore, agent that aims at multi-event of the angiogenesis process will be more desirable in anti-angiogenic therapy. Given to the immeasurable chemical and biological diversity [26], marine environment may be a prolific resource for discovery of novel angiogenesis inhibitors. Fascaplysin, a natural product from marine sponge, is demonstrated an angiogenesis inhibitor with VEGF blockage property in our previous study. In this paper, we further investigated the anti-proliferation effects of fascaplysin on human umbilical vein endothelial cell, the most studied endothelial cell model. Results shown that fascaplysin arrested G1 cell cycle arrest and induced apoptosis in HUVEC cells in a dose- and time-dependent manner, in addition to blocking VEGF in the anti-angiogenesis process.

The G1/S restriction point is the most important checkpoint in the cell cycle regulation, controlling the passage of eukaryotic cells from preparing for DNA synthesis (G1) into the DNA synthesis (S) phase [27]. Our data indicated that the G1 arrest caused by fascaplysin in HUVEC was significant. This is consistent with the observations in response to fascaplysin treatment of tumor cells ($p16^-$, pRb^+), and normal cells ($p16^+$, pRb^+), which were proved relating to inhibition of CDK4 [9]. Furthermore, the suppression of HUVEC cell cycle in G1 is unduerlined by the fact that tumor cell lines BeL-7402 and Hela were not as susceptible as HUVEC to the G1 arrest effect of fascaplyis. Moderate G1 arrest was observed in a relatively high fascaplysin concentration (4 μM) in BeL-7402 (Table 1) comparing to that in HUVEC (2.6 μM), and Hela was failed to be arrested in G1 phase (Table 2) by contraries. Different tissue of origin and degree of tumor progression might be the factors that affected the G1 arrest effect of fascaplysin. Though the response of HUVEC can not represent all of endothelial cells in tumor, the inherently stable characteristic and low mutant rate of endothelial cells might make fascaplysin effective against most of them.

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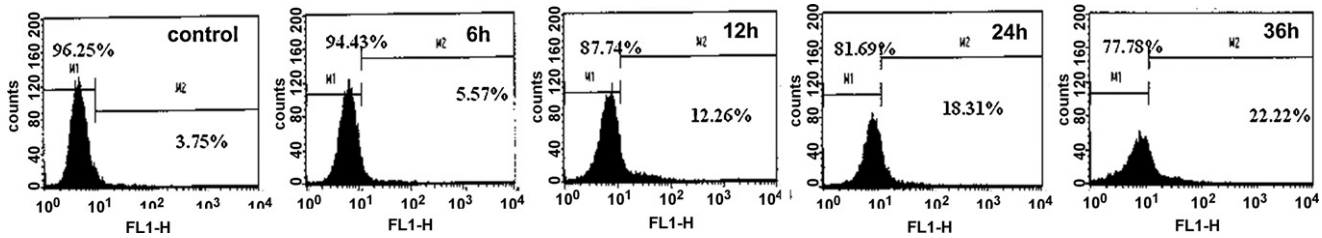

Fig. 6. Assessment of activated caspase-3. After treated with fascaplysin 1.3×10^{-6} M for 6, 12, 24 or 36 h, activated caspase-3 was detected using FITC-conjugated monoclonal active caspase-3 antibody and analyzed by flow cytometry. The percentages of active caspase-3 positive cells (M2) out of total number of counted cells (M1 + M2) were presented in the figures.

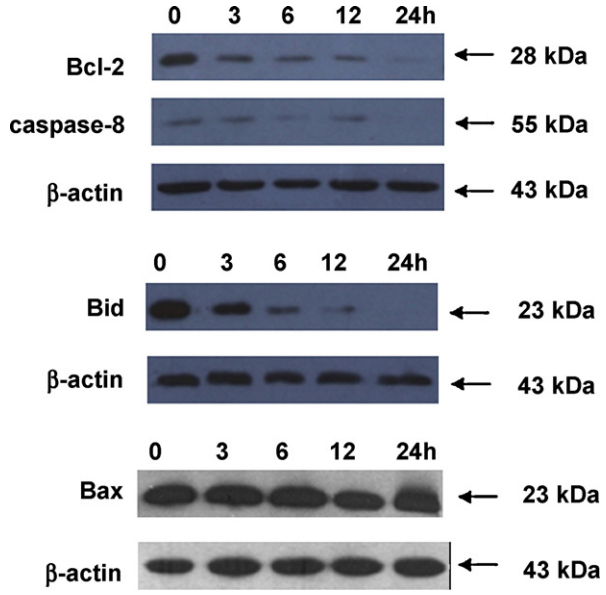

Fig. 7. Western blot analysis of apoptosis-related protein in fascaplysin-treated HUVEC cells. Cells were treated with 1.3×10^{-6} M of fascaplysin for 3, 6, 12, 24 h. The cellular proteins were extracted and separated by SDS-PAGE for immunoblot. β–actin was used as an internal control.

The current study indicated that fascaplysin-induced apoptosis has a greater consequence in the inhibition of HUVEC proliferation. The fascaplysin concentration that triggered apoptosis (1.3 μM) was lower than that caused G1 arrest (2.6 μM). Under this concentration condition, the mitochondrial pathway was revealed to be induced in the apoptosis event of HUVEC. It is known that the pro- and anti-apoptotic Bcl-2 family proteins are pivotal regulators of the mitochondrial pathway, controlling the irreversible cell death machinery. By modulating permeabilization of the inner and/or outer mitochondrial membranes, members of Bcl-2 family regulate the release of cytochrome *c* [28,29]. Previous reports have shown that the ratio of Bax to Bcl-2 determines the susceptibility of cells to death signals. Upregulation of Bax and downregulation of Bcl-2 [30], no change of the protein level of Bax [31], slight downregulation of Bax and downregulation of Bcl-2 [32], were demonstrated to cause apoptosis. Our data indicated that Bax protein level was increased slightly after 24 h, whereas Bcl-2 protein level gradually decreased in a time-dependent manner in fascaplysin-treated HUVEC cells. These results suggested that fascaplysin induced apoptosis through the mitochondrial pathway by shifting the Bax/Bcl-2 ratio in favour of apoptosis. The increasing level of active caspase-3 further supported this conclusion.

In summary, our present study showed more details about the potential role of fascaplysin in the anti-angiogenic therapy. Fascaplysin was found to inhibit the proliferation of HUVEC cells through inducing a G1 phase arrest, and apoptosis involving the mitochondrial pathway by the demonstration of induction of active caspase-3, and decrease of procaspase-8, Bid, and the ratio of Bax/Bcl-2. Additional studies are needed to determine other possible apoptosis signaling pathway that might be involved in the fascaplysin-induced HUVEC cells apoptosis.

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