

# Angiopoietin-1 is an apoptosis survival factor for endothelial cells

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**Abstract** We examined the effect of angiopoietin-1 (Ang1) on apoptosis in human umbilical vein endothelial cells (HUVECs). Ang1 (5–1000 ng/ml) dose-dependently inhibited apoptosis under a serum-deprived state. A significant apoptotic inhibition occurred with as low as 50 ng/ml. Two hundred ng/ml of Ang1 inhibited to approximately 50% of the control apoptotic rates for 96 h. Furthermore, an augmented antiapoptotic effect of Ang1 by the addition of 20 ng/ml vascular endothelial growth factor was observed. This Ang1-induced strong antiapoptotic effect in endothelial cells is a novel and intriguing finding and could be an additional description of Ang1-induced direct biological function.

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**Key words:** Angiopoietin; Apoptosis; Endothelial cell

## 1. Introduction

Vascular endothelium, a monolayer of cells lining the intima of the blood vessels is involved in a variety of functions, including coagulation, vascular permeability, vascular tonus and remodeling [1]. Endothelial cells, which are in direct contact with plasma and cellular components of blood, are the targets of many cytokines and growth factors [2,3].

Apoptosis, a strategic biologic process of eliminating unwanted cells is involved in the regulation of cell number under physiological and certain pathological conditions [4]. Apoptosis is associated with distinctive morphological and biological events, such as cellular shrinkage, nuclear condensation, and fragmentation of chromatin caused by cleavage endonuclease at the internucleosomal linker region, yielding mono- and polynucleosomal DNA fragments. Prevention of apoptosis in vascular endothelial cells by several growth factors and cytokines, such as the fibroblast growth factor [5], vascular endothelial growth factor (VEGF) [6], endothelin-1 [7] and adrenomedullin [8], have been studied. These molecules not only stimulate cell proliferation rate but also suppress apoptosis, thereby maintaining the surviving cell number.

The recent discovery of angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) has provided insights into the molecular and cellular mechanisms of blood vessel formation and maintenance [9,10]. Ang1 exerts its biological function through binding to the endothelial cell tyrosine kinase receptor, Tie2 [9,10]. In vivo analysis by targeted gene inactivation revealed that

Ang1 recruited and sustained periendothelial support cells [11], while Ang2 disrupted blood vessel formation in the developing embryo by antagonizing the effects of Ang1 on Tie2 [10]. Interestingly, transgenic overexpression of Ang1 [12] or gene transfer of Ang1 [13] increases vascularization in vivo. In vitro experiments demonstrated that Ang1 is weak for proliferation but is an effective molecule for sprouting [14] and chemotactic response [15,16] in endothelial cells. Although the above results suggest an important role of Ang1 in endothelial cells, its role as an apoptosis modulator has not been described carefully to date.

Here we examined the effect of Ang1 on apoptosis in cultured human umbilical vein endothelial cells (HUVECs). Our data indicate that Ang1 is a strong apoptosis survival factor in endothelial cells, and the addition of VEGF to Ang1 produced an augmenting effect for surviving apoptotic death.

## 2. Materials and methods

### 2.1. Cell culture and reagents

HUVECs and porcine pulmonary arterial endothelial cells (PPAECs) were prepared from human umbilical cords and porcine pulmonary arteries by collagenase digestion. The endothelial origin of the cultures was confirmed by the presence of factor VIII by immunofluorescence detection. HUVECs and PPAECs were maintained in M-199 medium supplemented with 20% (v/v) fetal bovine serum at 37°C in a 5% CO<sub>2</sub> atmosphere. The cells used in this study were from between passage 2 and 4. The recombinant human VEGF was purchased from R&D systems.

### 2.2. Production of Ang1 recombinant protein

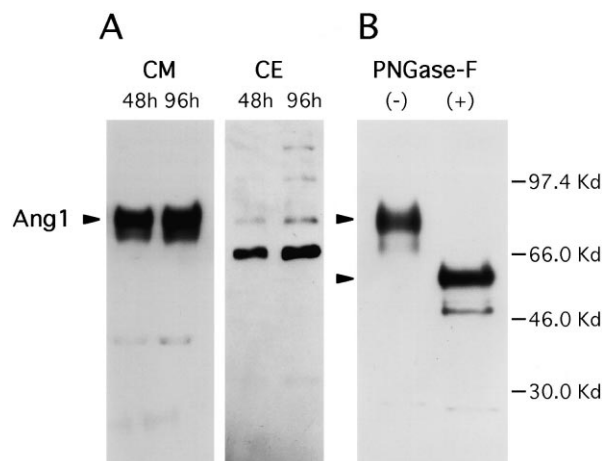
The full cDNA of human Ang1 was amplified from human adult heart cDNA, as a template, by PCR for 30 cycles at an annealing temperature of 52°C using sense and antisense primers [9]. The amplified DNA was cloned into the pCR-Blunt vector (Invitrogen) and sequenced. This human Ang1 cDNA was re-subcloned into the CMV promoter-driven mammalian cell expression vector, pcDNA3.1/Myc-His (Invitrogen), that has a DNA fragment (63 bp) encoding c-myc and a 6×His tag at the 3'-terminus of coding region as an open reading frame (CMV-Ang1-M-H). The CMV-Ang1-M-H gene construct was transfected into COS-7 cells using Lipofectamine Plus (Gibco BRL) and incubated at 37.5°C for 96 h in Dulbecco's modified Eagle's media (DMEM) with 2% fetal bovine serum under 5% CO<sub>2</sub>-95% atmosphere. To determine the presence of recombinant Ang1, the culture supernatant and cell lysates of COS-7 cells transfected with the CMV-Ang1-M-H were purified by Ni-NTA Spin Column (Qiagen) and were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system, and the proteins were electrotransferred to nitrocellulose membranes [15]. The nitrocellulose membranes were blocked by incubation in a blocking buffer, incubated with an anti-myc or anti-His antibody, washed, incubated with horseradish peroxidase conjugated secondary antibody, and signals were visualized by the ECL detection method according to the manufacturer's protocol (Amersham). Based on the above Western blotting result (Fig. 1), 1 l of the culture supernatant of COS-7 cells, transfected with the CMV-Ang1-M-H, were transferred to Ni-NTA Agarose Column (Qiagen) to obtain a fair amount of recombinant Ang1. The binding protein was eluted with the pH 8.0 buffer containing

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**Abbreviations:** VEGF, vascular endothelial growth factor; CMV, cytomegalovirus; cDNA, complementary deoxyribonucleic acid

Fig. 1. A: Detection of recombinant Ang1 protein from the culture medium and cell lysates of COS-7 cells transfected with CMV promoter driven mammalian cell expression vector containing the human Ang1 cDNA with the 3'-terminal extension encoding c-myc and 6×His tags. The culture medium and cell extracts of COS-7 cells were collected at 48 h and 96 h after transfection, and separated by SDS-polyacrylamide gel (10%) electrophoresis. Recombinant Ang1 protein was detected by Western blotting analysis with anti-myc antibody. CM, culture medium; CE, cell extracts. B: Comparison of molecular weights of recombinant Ang1 protein with (+) or without (–) PNGase-F treatment. Each lane contains 1.0 µg of purified human recombinant Ang1 protein, and Western blotted with an antibody to c-myc. The detailed procedures for purification and de-glycosylation are described in Section 2. Arrowheads indicate recombinant Ang1. Rainbow molecular marker (Amersham) was used as estimation of molecular mass.



250 mM imidazole. The elutes were dialyzed extensively against phosphate buffered saline (PBS) and were concentrated using Centricon 10 (Amicon) at 4°C. The salts were removed using the desalting column. Approximately 60 µg of recombinant Ang1 was obtained. The purity of this protein was determined by silver staining after SDS-PAGE. The glycosylation status of the recombinant Ang1 was determined with PNGase-F treatment according to the manufacturer's protocol (NEB).

### 2.3. Bioassay for recombinant Ang1 in endothelial cells

The biological activity of recombinant Ang1 protein was assayed by sprouting activity in PPAECs according to the studies described previously [14,17]. Briefly, PPAECs were grown to confluence on micro-

carrier (MC) beads (Sigma) and placed in a 2.5 mg/ml fibrinogen gel (Sigma) containing control buffer or various concentrations of recombinant Ang1, and 200 U/ml Trasylol (Bayer). Fibrin gels were incubated in M-199 containing control buffer or the same concentration of recombinant Ang1, 1.0% heat inactivated fetal calf serum and 200 U/ml Trasylol. After 2–3 days the extent of sprouting was determined using phase-contrast inverted microscope (Zeiss). To detect the nuclei of sprouting endothelial cells from MC beads, the fibrin gel containing beads was washed with PBS, fixed for 30 min in 4.0% paraformaldehyde, stained with Hoechst 33425 for 30 min and observed using a fluorescence microscope (Zeiss).

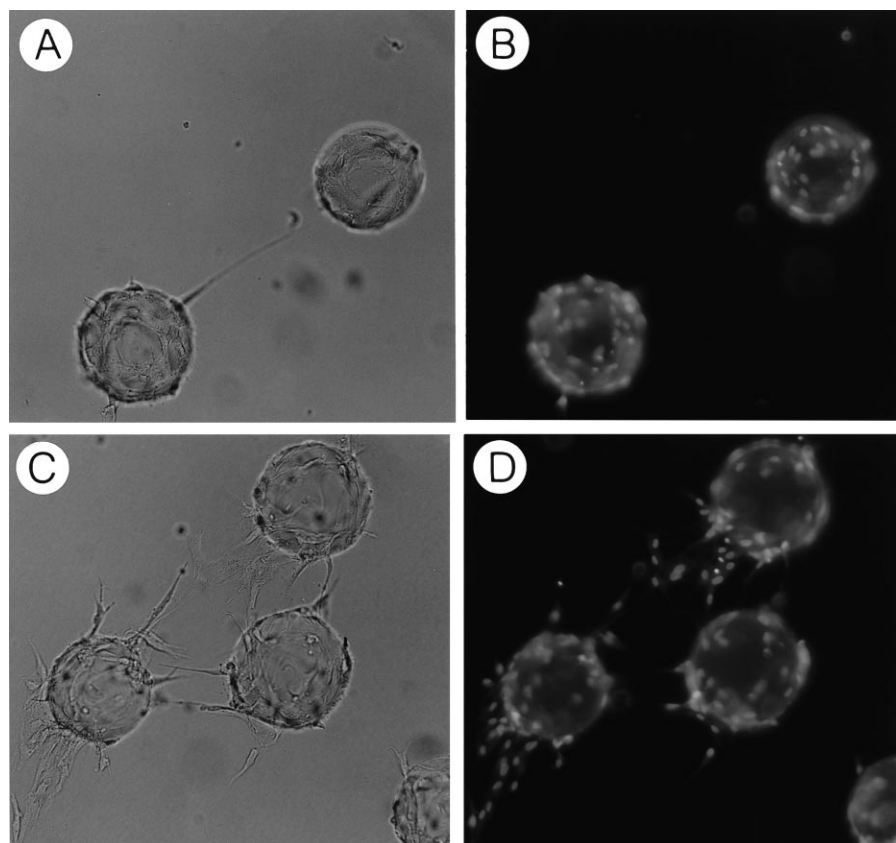


Fig. 2. Sprouting activity of recombinant Ang1 protein in PPAECs grown on microcarrier (MC) beads. The extent of sprouting is determined using phase-contrast (for bright field: A and C) and fluorescence (for Hoechst 33425 staining: B and D) inverted microscope (Zeiss). A and B: control; C and D: 200 ng/ml of recombinant human Ang1 protein for 3 days. Note that no sprouting is observed in control, but active sproutings are observed in the presence of Ang1. Fluorescence photographs reveal that most nuclei of endothelial cells are located on beads in control (B), whereas most nuclei of endothelial cells have migrated from beads in the presence of Ang1 (D). All magnifications are ×150. The detailed procedures for fixation and staining are described in Section 2.

#### 2.4. Quantitative determination of apoptosis

HUVECs were plated onto gelatinized 24-well plates ( $5 \times 10^4$  cells per well) in M-199 medium supplemented with 20% (v/v) fetal bovine serum and incubated for 24 h. The wells were extensively washed with PBS, and the medium was changed to serum-free M-199 medium containing various concentrations of recombinant Ang1, VEGF (20 ng/ml) or Ang1 (200 ng/ml) plus VEGF (20 ng/ml) as indicated in the text. Control cultures received serum-free M-199 medium with the same amount of PBS. All floating cells were collected with two PBS washes, and all adherent cells were also collected after trypsinization. The numbers and size distributions of the floating and adherent cells were determined with a Coulter Model ZF Particle Counter. To detect the apoptotic cells in the adherent cells, the parallel wells were washed with 0.9% sodium chloride, fixed for 15 min with 0.5% glutaraldehyde, and stained with Hoechst 33425 and Sytox green nucleic acid stainings. Apoptotic cells were counted by three independent, blinded investigators, in four different random locations using fluorescence microscopes (Zeiss). The number of apoptotic cells in the adherent cells was confirmed by CytoDeath kit according to the manufacturer's protocol (Boehringer Mannheim) in the part of this experiment. Both the nuclear stainings and the CytoDeath kit gave similar results. Therefore, in this study the total apoptotic events were presented as a percentage of the apoptotic (determined by nuclear staining) and floating cells in a given cell population.

### 3. Results and discussion

#### 3.1. Production of Ang1 recombinant protein

Ang1 has characteristic protein structures that contain a coiled-coil domain in the NH<sub>2</sub>-terminal portion and a fibrinogen-like domain in the COOH-terminal portion [9]. Ang1 is known to be a glycosylated secretory protein from the peri-

cytes and vascular smooth cells around endothelial cells [9]. A major band of  $\sim 75$  kDa was detected mainly in the culture supernatant, but not in the cell lysate of COS-7 cells after transfection (Fig. 1). These data indicated that the recombinant Ang1 was efficiently secreted from transfected cells. One liter of the culture supernatant of COS-7 cells 96 h after transfection and the purification using Ni-NTA Agarose Column provided us with approximately 60  $\mu$ g of recombinant human Ang1. The silver gel staining of the purified recombinant Ang1 after SDS-PAGE revealed a single band of  $\sim 75$  kDa and a purity of approximately 94% (data not shown). The observed molecular mass of the major band was larger than the calculated molecular mass of recombinant Ang1 ( $\sim 58$  kDa) since Ang1 contained several potential glycosylation sites as previously described [9]. Therefore, our recombinant Ang1 was treated with PGNase-F and Western blotted with an antibody to c-myc to examine the glycosylation status. Deglycosylation reduced the apparent molecular weight of recombinant Ang1 from  $\sim 75$  kDa to the size predicted from the amino acid sequence, 58 kDa (Fig. 1). These results indicate that our recombinant Ang1 from transfected COS-7 cells is an efficiently secreted glycoprotein.

#### 3.2. Bioassay for recombinant Ang1 in endothelial cells

Ang1 is weak for proliferation but is an effective molecule for sprouting [14] and chemotactic response [15] in endothelial cells in vitro. Therefore, the biological activity of the recombinant Ang1 protein was determined by sprouting activity in

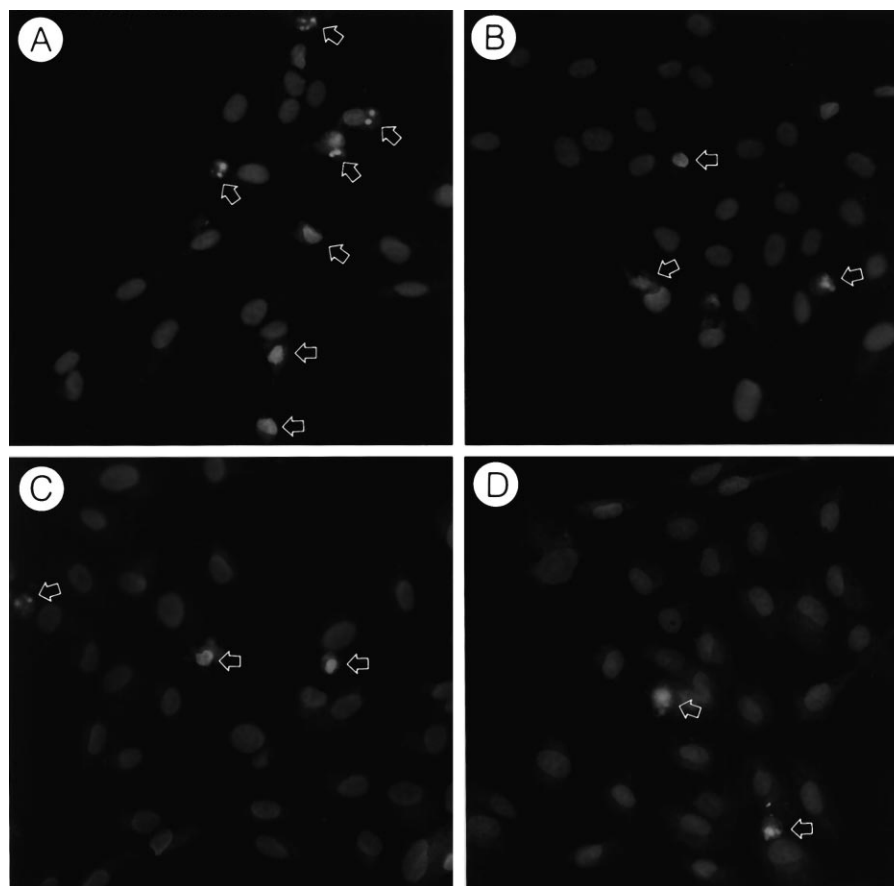


Fig. 3. Detection of apoptotic cells by morphological analysis of Sytox Green nucleic acid-stained nuclei. HUVECs, deprived of serum and incubated for 24 h without (A) and with VEGF (20 ng/ml) (B), Ang1 (200 ng/ml) (C) or VEGF (20 ng/ml) plus Ang1 (200 ng/ml) (D). Arrows indicate the apoptotic cells. All magnifications are  $\times 260$ . The detailed procedures for fixation and staining are described in Section 2.

PPAECs. 200 ng/ml of recombinant Ang1 produced an active sprouting activity, while the control buffer did not produce sprouting activity (Fig. 2). This endothelial sprouting activity was induced in a dose-dependent manner within a range of 10–1000 ng/ml (Kim et al., unpublished observation). These results were consistent with the previous study [14]. Thus, our purified recombinant Ang1 was biologically active in endothelial cells.

### 3.3. Ang1 is an apoptosis survival factor for endothelial cells

Cultured human endothelial cells have been demonstrated to undergo apoptosis under serum deprivation and apoptotic cells were detached from culture plates [18]. Therefore, we used the serum deprivation method for inducing an apoptosis in HUVECs. The percent age of apoptotic cells increased from 1.5% before serum deprivation to 11.3%, 39.3%, 77.6%, 93.0% and 96.3% at 12 h, 24 h, 48 h, 72 h and 96 h after serum deprivation (Figs. 3 and 4). Thus, the HUVECs underwent an active apoptosis during the initial 48 h after serum deprivation. 20 ng/ml of VEGF inhibited to approximately 55–60% of control the apoptotic rate in HUVECs for 48 h in the serum-deprived state (Figs. 3 and 4). These results were similar to others reported [6,19]. Notably, 200 ng/ml of Ang1 also inhibited to approximately 55–60% of control the apoptotic rate in HUVECs for 48 h under serum-deprived state (Figs. 3 and 4). Thus, VEGF and Ang1 produced similar antiapoptotic results in endothelial cells although the inhibitory doses of 50% were 10 times different. In addition, we determined the proliferation assay with Ang1 (200 ng/ml) using [<sup>3</sup>H]thymidine incorporated into HUVECs in the absence and presence of 10% serum. Under both conditions, Ang1 did not change the proliferative activity in HUVECs (data not shown) as in other reports [9,14]. Therefore, the Ang1-induced antiapoptotic effect resulted from the cell survival but did not result from the cell proliferation.

Furthermore, Ang1 (5–1000 ng/ml) dose-dependently inhibited the apoptosis in HUVECs at 24 h in the serum-deprived state (Fig. 4). A significant apoptotic inhibition was induced with as low as 50 ng/ml and a maximal antiapoptotic effect ( $64.4 \pm 10.7\%$  survival of control apoptotic cells) was induced with 1000 ng/ml of Ang1. These data suggest that the Ang1-induced antiapoptotic effect in endothelial cells may be mediated by its specific receptor, Tie2, binding. On the other hand, Ang2 is a naturally occurring antagonist of Ang1 that competes for binding to Tie2 and blocks Ang1-induced Tie2 autophosphorylation [10]. Therefore, it is worth examining whether Ang2 blocks the Ang1 induced antiapoptotic effect in endothelial cells through a Tie2 receptor. Furthermore, the intracellular mechanism of Ang1 responsible for the antiapoptotic effect remains to be elucidated.

The augmented antiapoptotic effect of Ang1 by addition of VEGF was observed during serum deprivation in HUVECs (Fig. 4). The addition of 20 ng/ml VEGF to 200 ng/ml Ang1 inhibited to approximately 75% of the control apoptotic rate for 96 h. Similar to our results, the modulatory role of VEGF on Ang1 has been observed in a mouse corneal micropocket assay [20]. Ang1 failed to stimulate an angiogenic response when administered alone. However, when co-administered with VEGF, Ang1 augmented the formation of neovessels.

While Ang1 and VEGF are mainly synthesized from periendothelial cells including vascular smooth muscle [9–11,20], their receptors are mainly located in the endothelial cells dur-

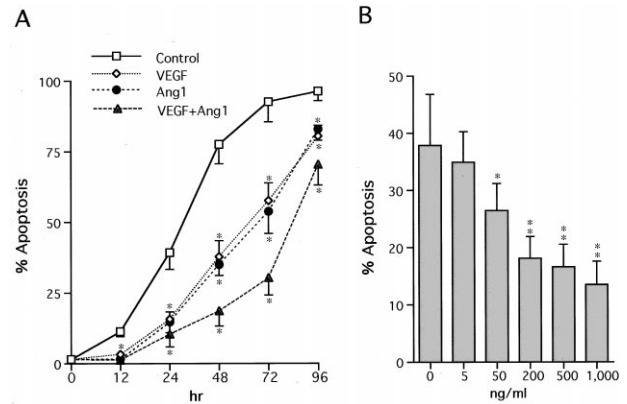


Fig. 4. Ang1 inhibits apoptosis in HUVECs. A: Time course of apoptosis after serum deprivation in the absence and presence of VEGF (20 ng/ml), Ang1 (200 ng/ml) or VEGF (20 ng/ml) (D) plus Ang1 (200 ng/ml). B: Dose-response effect of Ang1 on apoptosis in HUVECs at 24 h after serum deprivation. The detailed description for calculation of “% apoptosis” is described in Section 2. The points and bars show the mean  $\pm$  S.D. of six independent experiments. Statistical analysis between the values of control and the values of VEGF, Ang1, or VEGF plus Ang1 were performed using the Student *t*-test (\**P* < 0.01 in A; \**P* < 0.05, \*\**P* < 0.01 in B).

ing embryonic development and the adult period [20,21]. Although the regulators for VEGF expression are well characterized the regulators for Ang1 remain to be further elucidated. Ang1 seems not to be upregulated by hypoxia [22]. However, enhanced expression of Ang1 and Tie2 has been observed in glioblastoma, suggesting that Ang1 could be involved in tumor angiogenesis [23]. Importantly, Ang1 and Tie2 are expressed in normal adult vessels in which vasculogenesis or angiogenesis does not occur [10,11,21]. These constitutive expressions of Ang1 and Tie2 may be involved in apoptosis survival for the maintenance of endothelial cell integrity.

In summary, we examined the effect of Ang1 on apoptosis in cultured HUVECs. Our data indicate that Ang1 is a strong apoptosis survival factor in endothelial cells. This Ang1-induced antiapoptotic effect in endothelial cells is a novel and intriguing finding and could be an additional description of the Ang1-induced direct biological function.

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