Angiopoietin-1 Inhibits Irradiation- and Mannitol-Induced Apoptosis in Endothelial Cells

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Background and Purpose—Angiopoietin-1 (Ang1) is a vasculogenic factor that signals through the endothelial cell–specific Tie2 receptor tyrosine kinase. We recently reported that Ang1 prevented apoptosis induced by serum deprivation in endothelial cells. In this study, we examined whether Ang1 prevents apoptosis in endothelial cells treated with irradiation or clinical concentrations of mannitol.

Methods and Results—Ang1 prevented irradiation- and mannitol-induced apoptosis in human umbilical vein endothelial cells in a dose-dependent manner. Pretreatment with soluble Tie2 receptor, but not Tie1 receptor, blocked the antiapoptotic effect of Ang1. Two phosphatidylinositol 3-kinase (PI3-kinase)–specific inhibitors, wortmannin and LY294002, blocked the Ang1-induced antiapoptotic effect. The antiapoptotic potency of Ang1 was similar to or greater than that of vascular endothelial growth factor, basic fibroblast growth factor, and endothelin-1. Ang1 also prevented apoptosis in cultured endothelial cells from porcine pulmonary and coronary arteries and in endothelial cells of explanted rat aorta.

Conclusions—Ang1 promotes the survival of endothelial cells in irradiation- and mannitol-induced apoptosis through Tie2 receptor binding and PI3-kinase activation. Pretreatment with Ang1 could be beneficial in maintaining normal endothelial cell integrity during intracoronary irradiation or systemic mannitol therapy. (Circulation. 2000;101:2317-2324.)

Key Words: angiopoietin • endothelium • cells • apoptosis • radiation • mannitol

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 are in direct contact with the plasma and the cellular components of the blood and under certain pathological conditions are the targets of various noxious stimuli, such as toxins, drugs, and physical agents.2 Damage to the endothelium is a side effect of some types of therapy. Restenosis after coronary angioplasty is a major limitation in the treatment of atherosclerotic coronary artery disease.3 Intracoronary irradiation is a new and promising technique to prevent restenosis after angioplasty.4 However, irradiation induces apoptosis in endothelial cells,3 thereby inducing vasomotor dysfunction6 or reducing reendothelialization.7 Hyperosmotic mannitol therapy is widely used for reducing brain edema.8,9 However, clinical concentrations of mannitol induce apoptosis in endothelial cells, thereby damaging the vascular endothelium.10 Apoptosis induced by serum deprivation or irradiation in vascular endothelial cells can be prevented by several growth factors and cytokines, such as basic fibroblast growth factor (bFGF),5 vascular endothelial growth factor (VEGF),11 and endothelin-1 (ET-1).12 These molecules not only stimulate cell proliferation but also suppress apoptosis, thereby maintaining cell number.

Angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) have recently been identified as ligands of the endothelial cell–specific Tie2 receptor.13,14 In vivo analysis by targeted gene inactivation reveals that Ang1 recruits and sustains periendothelial support cells.15 Ang2 disrupts blood vessel formation in the developing embryo by antagonizing the effects of Ang1 on Tie2.14 Interestingly, transgenic overexpression16 or gene transfer17 of Ang1 increases vascularization in vivo. In vitro experiments have shown that Ang1 has specific effects on endothelial cells: it has little effect on proliferation, but it potently induces sprouting,18 chemotactic response,19 and network formation.20 Also, Ang1 is a strong apoptosis survival factor in endothelial cells under serum deprivation.20–22 Because Ang1 does not cause proliferation in endothelial cells,13,18 it could be a clinically useful endothelial protective factor.

In this study, we found that Ang1 prevents irradiation- and mannitol-induced apoptosis in endothelial cells through Tie2...
receptor binding and phosphatidylinositol 3'-kinase (PI3-kinase) activation. These data suggest that pretreatment with Ang1 may help maintain normal endothelial cell integrity during intracoronary irradiation and systemic mannitol therapy.

Methods

Materials
We obtained Ang1*, soluble Tie1 receptor-Fc (rTie1-Fc), and Tie2 receptor-Fc (rTie2-Fc) fusion proteins from Regeneron Pharmaceuticals, Inc. Ang1* is a recombinant version of Ang1 with modified
NH₂-terminus and mutated Cys²⁴⁵. Ang¹* is easier than Ang¹ to produce and purify. Mutation of the Cys²⁴⁵ in Ang¹, which is not shared between Ang¹ and Ang², does not alter its agonistic properties. The biological activities of recombinant Ang¹ and Ang¹* are similar, as confirmed by their high-affinity binding to and stimulation of the Tie² receptor in vitro. The Ang¹* recombinant protein was produced from Chinese hamster ovary (CHO) cells and affinity-purified with rTie²-Fc–conjugated Sepharose according to the method described by Maisonpierre et al. The purity of the protein was 95%, as judged by reducing and nonreducing silver-stained SDS-PAGE. rTie¹-Fc and rTie²-Fc fusion proteins were constructed, produced, and purified according to the method described by Davis et al. Recombinant human VEGF₁₆₅ and bFGF were purchased from R&D systems. Media and serum were obtained from Life Technology, Inc. Most other biochemical reagents, including mannitol, angiotensin II, ET-1, gelatin, antibiotics, antimycotics, and trypsin-EDTA and antibodies were purchased from Sigma, unless otherwise specified.

Cell Culture
Human umbilical vein endothelial cells (HUVECs) and porcine pulmonary arterial endothelial cells (PPAECs) were prepared from human umbilical cords and porcine pulmonary arteries by collagenase digestion as previously described. To obtain porcine coronary arterial endothelial cells (PCASMCs), pig hearts were delivered within 1 hour of slaughter under sterile conditions. A 5- to 7-cm section of coronary artery, from the initiation area of the left coronary artery to the midportion of the anterior interventricular artery, was carefully isolated. Isolated arteries were washed 3 times with PBS containing streptomycin, penicillin, and antimycotics and incubated with PBS containing 0.2% collagenase (type II; Worthington) for 15 minutes at room temperature. The arteries were transferred onto a sterile matrix, dissected sagittally, and fixed along the edges with small pins. The luminal areas were rubbed gently with surgical spears (No. 400101, Merocel Corp). The spears were dipped into PBS and swirled 5 to 6 times to detach the endothelial cells. The detached cells were collected and seeded onto a 3.5-cm gelatin-coated dish. The cells reached confluence, they were replated onto gelatin-coated dishes at split ratios of 1:2 or 1:3. Porcine coronary artery smooth muscle cells (PCASMCs) were prepared from a medial portion of porcine coronary artery by digestion with collagenase and elastase (Worthington) in the presence of soybean trypsin inhibitor. The endothelial or muscle origin of the cultures was confirmed by immunofluorescent detection of von Willebrand factor or smooth muscle actin, respectively: acceptable cultures had >95% cells positive to the corresponding antibody. These endothelial cells were maintained in M199 medium supplemented with 20% (vol/vol) heat-inactivated FBS, and PCASMCs were maintained in DMEM with 10% (vol/vol) FBS at 37°C in 5% CO₂. The primary cultured cells used in this study were between passages 2 and 4.

Induction of Apoptosis in Endothelial Cells
Endothelial cells were plated onto gelatinized 24-well plates (5 × 10⁴ cells per well) in medium containing 5% serum and were incubated for 24 hours. To produce irradiation-induced apoptosis, the cells were irradiated with a linear accelerator (Siemens, 6-MV x-ray, 2 Gy/min). To produce mannitol-induced apoptosis, the cells were grown to confluence in medium containing 20% serum, then switched to medium with 2% serum for 24 hours before the addition of various concentrations of mannitol. The final osmolarities in the medium were confirmed by an osmometer (Advanced Instruments).

Quantitative Determination of Apoptosis
Quantitative determination of apoptosis in cultured endothelial cells was performed as described previously. Floating apoptotic cells were collected with 2 PBS washes; adherent cells were collected by trypsinization. The numbers and size distributions of the floating and adherent cells were determined with a Coulter model Z1 Dual Counter System. To quantify the apoptotic cells among the adherent cells, the cells in a parallel well were washed with 0.9% sodium chloride, fixed...
for 15 minutes with 0.5% glutaraldehyde, and stained with Sytox green (Molecular Probes, Inc). Two independent, blinded investigators counted the apoptotic adherent cells identified by nuclear staining with a fluorescence microscope (Zeiss). Approximately 250 cells were counted in each of 4 different random locations. Interinvestigator variation was ≤5%. The mean number from the 2 investigators was used to estimate the percentage of apoptotic adherent cells. In some cases, we confirmed our data on the number of apoptotic cells among the adherent cells using the CytoDeath kit according to the manufacturer’s protocol (Boehringer Mannheim). The nuclear staining and the CytoDeath kit gave similar results. More than 97% of floating cells were apoptotic, as confirmed by both Sytox green staining and terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) assay (Oncor). Therefore, the percentage of apoptotic cells is based on the sum of the floating cells plus the apoptotic adherent cells in a given cell population.

Application of Ang1*, Soluble Tie Receptors, and PI3-Kinase Inhibitors to Endothelial Cells

Growth factor or cytokine was added to the cell medium 30 minutes before irradiation or mannitol treatment. Immediately after irradiation, cells were washed, then given fresh medium with fresh growth factor or cytokine. For mannitol treatment, the cells were washed, then given fresh medium containing the mannitol and fresh growth factor or cytokine. An ~5-fold molar excess of rTie2-Fc completely inhibits Ang1*-induced Tie2 phosphorylation in an in vitro assay (data not shown). Therefore, a 5-fold molar excess of rTie1-Fc or rTie2-Fc (2 μg/mL) was added 30 minutes before treatment with Ang1* (200 ng/mL). In the assay of PI3-kinase inhibitors, wortmannin (30 nmol/L; RBI, Inc) or LY294002 (100 nmol/L; RBI, Inc) was added 1 hour before treatment with Ang1* (200 ng/mL). The same amount of rTie1-Fc, rTie2-Fc, wortmannin, or LY294002 was freshly added to the medium after a wash, at the same time as the addition of Ang1. Control cultures received the same amount of the appropriate buffer or DMSO.

Explant Culture and Detection of Apoptotic Cells in the Endothelium of Rat Aorta

Explant culture of rat aorta was performed according to the method described by Merrick et al.23 Abdominal aortas were excised from male Sprague-Dawley rats. The aortas were cut into 5-mm ring segments and washed in PBS. The rings were cultured in M199 containing 2% FBS and treated with mannitol (300 mOsm) in the absence and presence of growth factor or cytokine as described above for 6 hours at 37°C, 5% CO2. The rings were washed in PBS, fixed in 10% neutral formalin, and embedded in paraffin. Tissue blocks were sectioned at 8 μm. An anti-CD31 monoclonal antibody (clone JC70A, DAKO) was used for endothelial staining, and the TUNEL method was used for detection of apoptotic cells. The endothelial cells and apoptotic cells in the endothelium of the aortic rings were viewed, counted, and photographed with a microscope (Axioskop, Zeiss) equipped with color CCD camera and monitor. Five to 6 aortic rings from different animals were used for each group. Approximately 100 to 120 endothelial cells were counted from 1 section of each ring. Thus, ~500 to 600 endothelial cells were counted per group.

Statistics

Data are expressed as mean±SD. Statistical significance between 2 groups was tested with the unpaired Student’s t test. Statistical significance between ≥2 groups was tested by 1-way ANOVA followed by the Student-Newman-Keuls test. Statistical significance was set at P<0.05.

Results

Ang1* Prevents Irradiation- and Mannitol-Induced Apoptosis in HUVECs Through the Tie2 Receptor and PI3-Kinase

Both irradiation and mannitol treatment caused apoptosis in HUVECs, as evidenced by floating cells seen with phase-
endothelial cells through Tie2 receptor binding and PI3-kinase activation but not through the Tie1 receptor.

**Comparison of the Antiapoptotic Potency of Ang1 With Other Growth Factors and Cytokines**

We compared the antiapoptotic effect of Ang1 with that of other growth factors and cytokines in HUVECs. Preliminary experiments revealed that a maximal dose of Ang1 (400 ng/mL) produced a further but slight increase in antiapoptotic effect, whereas a maximal dose of VEGF (40 ng/mL), bFGF (10 ng/mL), ET-1 (5 × 10^{-7} mol/L), or angiotensin II (5 × 10^{-7} mol/L) had no further antiapoptotic effect beyond that of a submaximal dose. Therefore, for comparison, a submaximal dose of each growth factor or cytokine was applied. In irradiation-induced apoptosis, the antiapoptotic potency of Ang1 (200 ng/mL) was similar to that of VEGF (20 ng/mL) and bFGF (5 ng/mL) but was greater than that of ET-1 (10^{-7} mol/L), although this difference was not statistically significant (Figure 4A). In mannitol-induced apoptosis, the antiapoptotic potency of Ang1 was significantly greater than that of VEGF, bFGF, and ET-1 (Figure 4B). Angiotensin II (10^{-7} mol/L) did not produce a significant effect in either experiment (Figure 4). Similar results were obtained in PPAECs (data not shown). These data indicate that Ang1 is at least as effective as or more effective than other endothelial survival factors.

**Antiapoptotic Effect of Ang1 Is Observed in PPAECs and PCAECs but Not in PCASMCs**

Endothelial cells from different locations have different responses to growth factors and cytokines.24 Therefore, we examined the antiapoptotic effect of Ang1* in PPAECs and PCAECs. The microscopic appearance of PCEACs was similar to but distinct from that of HUVECs and PPAECs. The cultured PCAECs are rounder and smaller than HUVECs and PPAECs but show a typical cobblestone appearance. The mitotic response to VEGF in PCAECs was less than the response in HUVECs and PPAECs, whereas the sprouting response to Ang1* is very active in PCAECs, moderate in PCEACs, and very low in HUVECs (data not shown). Although the percentages of control apoptotic cells in PPAECs and HUVECs were similar, the percentage of apoptotic cells in PPAECs was ≈10% to 15% less than in HUVECs at 24 hours after 10-Gy irradiation (Figure 5A). However, Ang1* inhibited ≈30% to 35% of the irradiation-induced apoptosis in PPAECs (Figure 5A). The apoptotic and antiapoptotic responses to mannitol and Ang1* in PPAECs were similar to those in HUVECs (Figure 5B). Interestingly, PCAECs had little apoptosis 24 hours after irradiation or 6 hours after mannitol. However, the percentage of apoptotic cells in PCAECs 48 hours after irradiation or 12 hours after mannitol closely resembled those of PPAECs at shorter time points (Figure 5). In all cases, Ang1* inhibited ≈30% to 35% of the irradiation- or mannitol-induced apoptotic rate in PCAECs. In contrast, Ang1* did not produce a significant antiapoptotic response in PCASMCs, which lack the Tie2 receptor (Figure 5).
Ang1 Prevents Apoptosis in the Endothelial Cells of Explanted Rat Aorta

Aortic organ culture was used to study endothelial cell viability in a system with intact morphology and minimal cell division.23 Rings prepared from rat aorta were incubated with mannitol (300 mOsm) in the absence or presence of agent for 6 hours, and apoptosis was assessed by color detection with TUNEL labeling (Figure 6A). Incubation with buffer alone resulted in few TUNEL-positive endothelial cells (2.2 ± 0.6%), whereas mannitol caused an 8.7-fold increase in TUNEL-positive endothelial cells (Figure 6). Pretreatment with Ang1* decreased the TUNEL-positive endothelial cells by ~40% (Figure 6). This was significantly greater than the antiapoptotic potency of VEGF (22%), bFGF (13%), and ET-1 (5%) (Figure 6).

Discussion

Restenosis after coronary angioplasty is a major limitation in the treatment of atherosclerotic coronary artery disease.3 Intracoronary irradiation is a new and simple technique developed to prevent restenosis after angioplasty.4 In fact, a 2-year follow-up study of catheter-based radiotherapy for preventing coronary restenosis showed potentially significant clinical benefit.25 Nevertheless, intracoronary irradiation inevitably causes some injury to coronary endothelial cells.5–7 The integrity of endothelial cells and reendothelialization around areas injured by angioplasty or irradiation are essential for preventing restenosis.26 Our study indicates that Ang1 inhibits irradiation-induced apoptosis in coronary artery endothelial cells. These data suggest that pretreatment with Ang1 could prevent apoptosis in coronary artery endothelial cells during intracoronary irradiation, potentially reducing the restenosis rate.

Mannitol is a cell-impermeant, nonmetabolizable sugar administered intravenously as a hypertonic solution for treating brain edema.8,9 Mannitol is customarily administered every 3 to 4 hours in bolus doses of up to 1.5 g/kg body wt, resulting in a final serum osmolarity of 320 mOsm.27 The beneficial clinical effects of mannitol have been attributed to the consequent increase in intravascular volume and coincident decrease in extracellular fluid volume.8,9 However, mannitol may activate inflammatory mediators and adversely affect certain cell types.28 Clinical concentrations of mannitol activate tyrosine and stress kinases and induce apoptosis in bovine aortic endothelial cells.10 Thus, the clinical use of mannitol may exert direct deleterious effects on vascular endothelium. In this study, a clinical concentration of mannitol increased apoptosis in 3 different endothelial cell cultures and in the endothelial cells of explanted rat aorta. Thus, we conclude that Ang1 inhibits mannitol-induced apoptosis in the endothelial cells. This finding suggests that pretreatment with Ang1 could prevent apoptosis in endothelial cells during mannitol therapy, thereby reducing the deleterious effect on endothelial cells.

Our results indicate that the anti-apoptotic effect of Ang1 in endothelial cells is mediated through binding to the Tie2 receptor in both irradiation- and mannitol-induced apoptosis. Other reports noted that soluble Tie2 receptor, but not Tie1 receptor, could block Ang1-induced sprouting,18 chemotactic effects,19 and network formation20 in endothelial cells. The intracellular second messenger signal transduction cascades initiated by Ang1-Tie2 binding are not well characterized. A recent report indicated that Tie2 activates PI3-kinase and Akt.28 The PI3-kinase and Akt pathways are common features in the transduction of the anti-apoptotic effects of growth factors.30 Therefore, we examined whether the PI3-kinase
pathway was involved in the antiapoptotic effect of Ang1 in endothelial cells. Wortmannin or LY294002, 2 potent inhibitors of PI3-kinase, completely reversed the antiapoptotic effect of Ang1 on both irradiation- and mannitol-induced apoptosis. Therefore, PI3-kinase activation could be an essential step in the antiapoptotic effect of Ang1.

The present results show that the antiapoptotic potency of Ang1 in endothelial cells is higher than that of VEGF, bFGF, or ET-1 in mannitol-induced apoptosis. As we demonstrated, Ang1 prevents endothelial apoptosis through the Tie2 receptor. VEGF is known to prevent endothelial apoptosis through the Flk1 receptor. Tie2 and Flk1 receptors are selectively localized in endothelial cells. Thus, Ang1 and VEGF are therapeutic candidates for preventing endothelial cell apoptosis under certain pathological conditions. However, VEGF exerts multiple effects, including proliferation and vascular permeability, whereas Ang1 exerts few cellular effects in the endothelial cells. Preliminary results of differential-display polymerase chain reaction revealed that VEGF increases ∼0.25% of mRNA transcripts, whereas Ang1 increases ∼0.04% of mRNA transcripts among the ∼25 000 mRNA transcripts of HUVECs (unpublished observations). Therefore, Ang1 could be better than VEGF in terms of clinical safety.

In conclusion, the present data suggest that pretreatment with Ang1 may provide a beneficial effect in maintaining normal endothelial cell integrity during intracoronary irradiation and mannitol therapy through the antiapoptotic effect of Ang1 in endothelial cells. However, further in vivo studies will need to be done before clinical application.

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