Endothelial Cell Surface ATP Synthase-Triggered Caspase-Apoptotic Pathway Is Essential for K1-5-Induced Antiangiogenesis

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ABSTRACT
We have recently reported the identification of kringle 1-5 (K1-5) of plasminogen as a potent and specific inhibitor of angiogenesis and tumor growth. Here, we show that K1-5 bound to endothelial cell surface ATP synthase and triggered caspase-mediated endothelial cell apoptosis. Induction of endothelial apoptosis involved sequential activation of caspases-8, -9, and -3. Administration of neutralizing antibodies directed against the α- and β-subunits of ATP synthase to endothelial cells attenuated activation of these caspases. Furthermore, inhibitors of caspases-3, -8, and -9 also remarkably blocked K1-5-induced endothelial cell apoptosis and antiangiogenic responses. In a mouse tumor model, we show that caspase-3 inhibitors abolished the antitumor activity of K1-5 by protecting the tumor vasculature undergoing apoptosis. These results suggest that the specificity of the antiendothelial effect of K1-5 is attributable, at least in part, to its interaction with the endothelial cell surface ATP synthase and that the caspase-mediated endothelial apoptosis is essential for the antiangiogenic activity of K1-5. Thus, our findings provide a mechanistic insight with respect to the antiangiostatic action and signaling pathway of K1-5 and angiostatin.

INTRODUCTION
Endogenous angiogenesis inhibitors produced by various tissues are critical to maintain the quiescent state of the vasculature in most adult tissues and organs (1). Although it is believed that production of these inhibitors is decreased in actively angiogenic tissues, they are often found in tumor tissues or in association with tumor growth, e.g., angiostatin, endostatin, tumstatin, serpin antithrombin, and thrombospondin-1 are present in various tumor tissues (2–6). These inhibitors can be produced by tumor cells and/or host cells infiltrated in the tumor (7). Switching on the expression of these inhibitors at high levels is perhaps one of the host defense mechanisms to prevent tumor growth. In most cases, these angiogenesis inhibitors may suppress tumor growth and keep tumors in a microscopic dormant state by preventing essential neovascularization (8, 9). However, most visible tumors switch on their angiogenic phenotypes by overexpression of angiogenic factors, including the families of vascular endothelial growth factor/vascular permeability factor, fibroblast growth factor-2, and angiopeptin (1, 10–14). Almost all angiogenesis inhibitors have been tested or at least considered as therapeutic agents in the treatment of tumors (8, 9). Although it is believed that production of angiostatic activity of K1-5 is contained within the first three kringle domains of plasminogen (2). Most angiostatic activity of angiostatin is currently in clinical trials for the treatment of primary tumors and metastasis, the underlying molecular mechanisms and signaling pathways of angiostatin-mediated angiostatic activity remain poorly understood. The structure of angiostatin includes the first three or four kringle domains of plasminogen (2). Most angiostatic activity of angiostatin is contained within the first three kringle domains of plasminogen (2). Most angiostatic activity of angiostatin is contained within the first three kringle domains of plasminogen (2). Most angiostatic activity of angiostatin is contained within the first three kringle domains of plasminogen (2). Most angiostatic activity of angiostatin is contained within the first three kringle domains of plasminogen (2). Most angiostatic activity of angiostatin is contained within the first three kringle domains of plasminogen (2). Most angiostatic activity of angiostatin is contained within the first three kringle domains of plasminogen (2).

MATERIALS AND METHODS

Reagents, Cells, and Animals. Human plasminogen, K1-3, and K1-5 were purified and purified to homogeneity according to our methods published previously (17). An anticaspase-3 monoclonal antibody was obtained as a gift (In Vitro, Stockholm, Sweden). Cell membrane permeable caspase-3 inhibitors, z-DEVd-fmk, z-IETD-fmk, and Ac-DEVD-CHO (Enzyme System Products, Livermore, CA), cell permeable DEVD-CHO (Calbiochem, San Diego, CA), and Ac-DEVd-CHO (Sigma, Stockholm, Sweden) were purchased. The anti-endothelial cell surface ATP synthase α- or β-subunit antibodies were obtained from Molecular Probes (Eugene, OR). BCE cells were kindly provided by Dr. Judah Folkman (Harvard Medical School, Boston, MA) and maintained as described previously (17). Rat vascular smooth muscle cells (VSMC) (a kind gift from Dr. Johan Thyberg, Karolinska Institute, Stockholm, Sweden) were grown in 10% heat-inactivated FCS-F12-Ham’s medium and murine T241 fibrosarcoma cells, and Lewis Lung carcinoma cells were grown and assayed in 10% FCS-DMEM. Male 5–6-week-old C57BL/6 mice (Microbiology and Tumor Biology Center, Karolinska Institute) were acclimated and caged in groups of six or fewer. Animals were anesthetized with injections of hypnorm: dextrometorphan:H2 O 1:1:2 before all procedures and euthanized with a lethal dose of CO2. All animal studies were reviewed and approved by the animal care and use committee of the Stockholm Animal Board.

Fluorescence-Activated Cell Sorter Analysis. Proliferating BCE cells were treated with 1 μM K1-5 or plasminogen for 24 h. The cells were trypsinized and stained with fluorochrome-labeled inhibitors of caspases (FAM-VAD-FMK) according to manufacturer’s protocols (Immunochemistry Technologies, Bloomington, MN). Stained cells were analyzed with a Becton Dickinson FacsScan and CellQuest software.
Detection of Apoptotic Endothelial Cells and Caspase-3 Activation. Proliferating BCE cells were treated with K1-3, K1-5, or plasminogen (1 μM) in DMEM containing low glucose supplemented with 10% bovine calf serum for 24 h. Cells were harvested and resuspended in PBS with 30 mM glycerol and 0.1 M NaCl, followed by drying on slides and fixation with aceto- methanol (1:1) for 10 min. The fixed cells were stained with ethidium bromide or Hoescht 33258 (500 ng/ml). Apoptotic cells were counted in randomly selected fields using fluorescent microscopy (×20, 10 fields/sample). z-DEVD-fmk, z-IETD-fmk, and z-LEHD-fmk at 20 μM were incubated with the cells for 2 h before addition of K1-5. Apoptotic cells were detected under a fluorescent microscope. BCE cells, treated with cisplatin at 10 μM, K1-3, K1-5, or plasminogen at 1 μM, or various concentrations of K1-5 for 24 h, were lyzed with 1% Triton X-100, 20 mM Tris-HCL (pH 8.0), 15 mM NaCl, and 5 mM EDTA. In another experiment, K1-5 at 1 μM was analyzed at different time points. Equal amounts of protein from each sample were separated on 4–12% Bis-Tris gels (Invitrogen, Stockholm, Sweden). Detection of cleaved and activated caspase-3 by a specific monoclonal antibody was performed using the enhanced chemiluminescence plus system (Amersham Biosciences AB, Uppsala, Sweden).

In Vitro Caspase Assays. Caspase-3-like, caspase-8, and caspase-9 activities were fluorometrically determined by cleavage of cellular substrates of DEVD-7-amino-4-methyl coumarin (DEVD-AMC), IETD-AMC, or LEHD-AMC, respectively, according to a protocol described previously (Peptide Institute, Osaka, Japan; Ref. 28). Briefly, ∼0.5 × 10^6 cells were collected at each time point, washed twice in PBS, centrifuged by sedimentation, and kept at −20°C. Some samples were preincubated for 1 h at 37°C with z-DEVD-fmk, z-IETD-fmk, and z-LEHD-fmk (20 μM; Enzyme Systems Products), with 100 μg/ml anti-integrin-αvβ3-neutralizing antibody (Chemicon International, Inc., Temecula, CA) or 100 μg/ml anti-α- and/or anti-β-ATPase antibody (Molecular Probes). Frozen cells were resuspended in 25 μl of PBS before measurement and transferred onto a 96-well microtitration plate. The appropriate peptide substrates were mixed in a reaction buffer containing 100 mM 4-morpholinopropanesulfonic acid (pH 6.5; for caspase-9) or 100 mM HEPES (pH 7.0; for caspases-3 and -8), 10% polyethylene glycol, 0.1% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid, 5 mM DTT, and 10 μM NP40. The cleavages of the fluorogenic peptide substrates were monitored by AMC release in a Fluoroscan II plate reader (Labsystems, Stockholm, Sweden) using λ = 355 nm for excitation and 460 nm for emission. Fluorescence units were generated with free AMC. Data were analyzed by linear regression.

Immunofluorescence Analysis. BCE cells maintained on gelatinized glass coverslips were stained with the anti-ATP synthase antibodies as described previously (27). Briefly, BCE cells were washed with PBS several times before fixing with 4% paraformaldehyde. A sample was permeabilized with 100% ethanol for 5 min at room temperature as positive control. For immunofluorescent staining, cells were incubated with murine monoclonal antibodies against the α- or β-subunit of ATP synthase (Molecular Probes), followed by incubation with a horse antimouse IgG FITC (1:50; Vector Laboratories, Inc., Burlingame, CA). Samples stained only with the secondary antibody served as negative controls. After vigorous washing, cells were mounted and analyzed using a fluorescent microscope with ×40 magnification.

ELISA Assay. Purified recombinant F1 ATP synthase (15 μg/ml) expressed in Escherichia coli bacterial cells was passively adsorbed onto 96-well plates (Immulon 4HBX Flat Bottom Microfilter Plate, VWR International, Batavia, IL). Briefly, plates were coated with recombinant F1 ATP synthase in 50 μl of 0.1 M NaHCO3 (pH 9.6) and incubated overnight at 4°C. Nonspecific sites were blocked with PBS (pH 7.0) containing 1% BSA for 2 h at room temperature. Increasing amounts of K1-5 (0–0.7 μg) were added to a final volume of 50 μl. The samples were incubated at 4°C overnight, washed with 0.05% Tween 20 in PBS (pH 7.0), and then incubated with an antihuman angiostatin (goat IgG; R&D Systems, Inc., Minneapolis, MN) antibody for 4 h. After washing, the samples were incubated with biotin-rabbit antigen IgG (Zymed, San Francisco, CA) for 1 h, followed by incubation with horseradish peroxidase-conjugated streptavidin (Zymed) for 1 h. After a final wash, 100 μl of 3,3’,5,5’-tetramethylbenzidine (TMB) substrate were added to each well, and the reaction was terminated with 100 μl of 1 M H2SO4. The absorbance of each sample at λ = 450 nm was determined with a Molecular Devices SpectraMax Plus-384 plate reader (n = 4).

Measurement of F1 ATPase Activity. F1 ATPase activity was measured according to methods described previously (29). The assay exhibits activity only in the reverse direction, acting to hydrolyze ATP. The hydrolysis of ATP to ADP is coupled to the oxidation of NADH via pyruvate kinase and lactate dehydrogenase. The oxidation of NADH to NAD is read at λ = 340 nm as a decrease in absorbance. Therefore, a decrease in absorbance is a measure of F1 ATPase activity. Briefly, recombinant F1 ATP synthase was coincubated with K1-5 for 30 min before the initiation of the enzymatic reaction. Control studies were performed with azide or in the absence of purified recombinant F1 protein (n = 3).

Chick Embryo Chorioallantoic Membrane (CAM) Assay. The CAM assay was performed as described previously (17). Three-day-old fertilized white Leghorn eggs (OVA Production AB, Moringåva, Sweden) were cracked, and chick embryos with intact yolk sacs were carefully placed in 20 × 100-mm plastic Petri dishes. After 48-h incubation in 4% CO2 at 37°C, disks of methylcellulose containing K1-5 (12.5 μg/mesh) alone, or in combination with the cell permeable DEVD-CHO (1–10 μg/mesh), dried on a nylon mesh (4 × 4 mm) were implanted on the CAM of individual embryos. The nylon mesh disks were made by desiccation of 20 μl of 0.45% methylcellulose (in H2O). After 48–72 h of incubation, embryos and CAMs were examined for the formation of avascular zones in the field of the implanted disks using a stereo microscope. Six to nine embryos were used in each group. Values represent mean determinates ± SEM.

Mouse Corneal Micropocket Assay. The mouse corneal assay was performed according to procedures described previously (17, 30). Corneal micropockets were created with a modified von Graefe cataract knife in the eyes of male 6–7-week-old C57BL/6 mice. Micropockets (0.35 × 0.35 mm) of sucrose aluminum sulfate (Buhk Meditec, Copenhagen, Denmark) coated with hydron polymer type NCC containing −80 ng of fibroblast growth factor-2 alone, a mixture of fibroblast growth factor-2 and Ac-DEVD-CHO (2.5 μg/pellet), Ac-DEVD-CHO alone, or PBS were implanted into the corneal pockets, positioned −1.2 mm from the corneal limbus. Mice were treated with s.c. injections of either PBS or K1-5 at a dose of 2 mg/kg/day. Corneal neovascularization in each group (n = 10) was examined using a slit lamp microscope at day 5 after pellet implantation.

Tumor Studies. Approximately 1 × 10^6 murine T241 fibrosarcoma tumor cells were implanted s.c. into each C57BL/6 mouse. Six to seven mice were used in the treated and control groups. Intratrabecular injections with 50 μg of K1-5 alone or in combination with 10 μg of Ac-DEVD-CHO in 100 μl/mouse began shortly after tumor cell implantation and continued once daily for a total of 15 treatments. Visible primary tumors were measured using digital calipers on the days indicated. Tumor volumes were calculated according to the formula width^2 × length × 0.52 as reported previously (17).

Immunohistochemistry. For detection of apoptotic endothelial cells in the tumor vasculature, tumor tissues were fixed with 4% paraformaldehyde and embedded using a paraffin method. Sections (5 μm) were dewaxed and rehydrated according to standard protocols. The terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining was performed according to a modified fluorescein in situ Death Detection Kit (Amersham). Briefly, the samples, after deparaffinization and rehydration, were blocked using 3% H2O2 in methanol and treated with 20 μg/ml proteinase K (Invitrogen). TUNEL reaction mixture was incubated with samples in a humid atmosphere at 37°C for 1 h. To detect CD31-positive endothelial cells, the sections were blocked with 20% normal rabbit serum and by using avidin-biotin kit (Vector Laboratories). The sections were stained with a rat antimouse monoclonal CD31-biotin antibody at 1:100 (PharMingen, Stockholm, Sweden) and streptavidin conjugated to Cy3 (1:2500; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The sections were photographed and counted under a fluorescent microscope at ×40 magnification.

RESULTS

Induction of Endothelial Apoptosis by K1-5. We have reported previously that K1-5 potently inhibits BCE cell growth in vitro. To determine whether K1-5 induces endothelial cell apoptosis, purified proteolytically derived human K1-5 at a concentration of 1 μM, known to inhibit endothelial cell growth, was incubated with proliferating BCE cells (17). After 24-h incubation, a significant proportion
T241 cells were used as controls. Nontreated VSMC (M) and plasminogen-treated BCE cells. Cell morphology stained with Hoechst of K1-5-treated K1-5, and plasminogen-treated (L) BCE cells. Cell apoptosis, which likely involves a cascade of caspase activation.

Findings demonstrate that K1-5 specifically induced endothelial cell apoptosis, which is essential for the potent antiangiogenic activity of K1-5, a key mediator of K1-5-induced endothelial cell apoptosis. To test this hypothesis, we applied z-DEVD-fmk, a cell membrane permeable and irreversible caspase-3 inhibitor, to prevent K1-5-induced endothelial cell apoptosis. At a concentration of 20 μM, z-DEVD-fmk almost completely blocked K1-5-induced BCE cell apoptosis (Fig. 2, D–F). These results demonstrate that caspase-3 is an essential and key mediator of K1-5-induced endothelial cell apoptosis. Western blot analysis revealed that DEVD-fmk did not seem to affect processing and activation of caspase-3 induced by K1-5 (Fig. 2G). However, DEVD-fmk almost completely inhibited caspase-3 activity in K1-5-treated BCE cells (Fig. 2H).

**Prevention of Endothelial Apoptosis in Vitro by a Caspase-3 Inhibitor.** If activation of caspase-3 by K1-5 is essential for endothelial cell apoptosis, inhibition of caspase-3 should block K1-5-induced endothelial cell apoptosis. To test this hypothesis, we applied z-DEVD-fmk, as a cell membrane permeable and irreversible caspase-3 inhibitor, to prevent K1-5-induced endothelial cell apoptosis (36, 37). At a concentration of 20 μM, z-DEVD-fmk almost completely blocked K1-5-induced BCE cell apoptosis (Fig. 2, D–F; P < 0.001). These results demonstrate that caspase-3 is an essential and key mediator of K1-5-induced endothelial cell apoptosis. Western blot analysis revealed that DEVD-fmk did not seem to affect processing and activation of caspase-3 induced by K1-5 (Fig. 2G). However, DEVD-fmk almost completely inhibited caspase-3 activity in K1-5-treated BCE cells (Fig. 2H).

**Impairment of the in Vitro Angiostatic Activity of K1-5 by Caspase Inhibitors.** To demonstrate in vivo that caspase-3-mediated angiostatic activity is essential for the potent antiangiogenic activity of K1-5, a cell membrane permeable and irreversible caspase-3 inhibitor, DEVD-CHO, was coimplanted with K1-5 in the chick CAM assay. As expected, K1-5 at the dose of 12.5 μg/mesh induced the formation of avascular zones in all CAMs (n = 7). Interestingly, when K1-5 was combined with DEVD-CHO, the antiangiogenic effect was completely abolished. The opposing antiangiogenic effect was dose dependent and statistically significant (Fig. 2I).

To further investigate the inhibitory effect of DEVD-CHO on the antiangiogenic activity of K1-5, we performed the mouse corneal angiogenesis assay (17). The fibroblast growth factor-2-induced cor

**Fig. 1. Induction of endothelial apoptosis.** Kringle (K)1-3, K1-5, or plasminogen was incubated with BCE cells for 24 h, followed by staining cells with Hoechst (A–D) or ethidium bromide (E–H). Apoptotic cells were quantified using Hoechst staining and present as means in percentages (±SEM). **A**–**D**, P < 0.001. Fluorescence-activated cell sorter analysis of cell populations with activated caspases of nontreated (J), K1-5-treated (K), and plasminogen-treated (L) BCE cells. Cell apoptosis was dose dependent and statistically significant (Fig. 2, D–F; P < 0.001). These results demonstrate that caspase-3 is an essential and key mediator of K1-5-induced endothelial cell apoptosis. Western blot analysis revealed that DEVD-fmk did not seem to affect processing and activation of caspase-3 induced by K1-5 (Fig. 2G). However, DEVD-fmk almost completely inhibited caspase-3 activity in K1-5-treated BCE cells (Fig. 2H).
neal angiogenesis was almost completely inhibited by K1-5 treatments at the dose of 2 mg/kg/day (Fig. 2J). Consistent with the CAM assay, Ac-DEVD-CHO could completely neutralize the antiangiogenic activity of K1-5 in this model (Fig. 2K). Quantification of corneal neovascularization as vessel areas showed that the antagonistic effect of Ac-DEVD-CHO on the antiangiogenic activity of K1-5 was statistically significant (P < 0.001; Fig. 2L). These data demonstrate that K1-5-induced antiangiogenesis requires caspase-3-mediated apoptosis.

Neutralization of Antitumor Activity by Caspase Inhibitors Is Correlated with Prevention of Tumor Vasculature from Apoptosis.

We reported previously that administration of K1-5 exhibited potent antitumor activity in a mouse fibrosarcoma model (17). Consistent with this finding, administration of K1-5 into murine fibrosarcomas at the dose of 50 μg/mouse/day resulted in significant suppression of tumor growth (n = 6; Fig. 3A). Approximately 83% of the antitumor effect was observed at day 15 after treatment. In contrast, Ac-DEVD-CHO alone (10 μg/mouse/day) did not significantly inhibit tumor growth (Fig. 3A). The antitumor activity of K1-5 was totally attenuated when K1-5 and Ac-DEVD-CHO were coadministered into tumors (Fig. 3A). These results indicate that the antitumor activity of K1-5 requires caspase-3-mediated apoptosis.

To correlate the antitumor effect of K1-5 with its ability to induce microvessel apoptosis, we stained tumor tissues with endothelial- and apoptotic-specific markers. Tumor microvessels were stained with a specific anti-CD31 antibody and labeled with red color (Fig. 3B). Apoptotic cells in tumor tissues were revealed by TUNEL method labeled with green color. A significant reduction of tumor neovascularization was observed in K1-5-treated tumors (Fig. 3B). Again, Ac-DEVD-CHO only slightly affected overall tumor cell apoptosis in the K1-5-treated tumors as compared with K1-5 alone. A significant number of
In B, determinants were quantified under a microscope (were detected by superimposing two images using a digital program. All positive signals were measured every other day according to a standard formula: width² × length × 0.52. In I, tumor sections (day 15) were double stained with a specific antibody against CD31 (vessels in red) and TUNEL (apoptotic cells in green). Apoptotic microvessels (yellow) were detected by superimposing two images using a digital program. All positive signals were quantified under a microscope (×400, 15 fields), and data were represented as mean determinants (+SEM).

endothelial cells in tumor vessels underwent apoptosis in the K1-5-treated tumors as revealed by CD31/TUNEL double staining of the same sections (arrows, yellow in Fig. 3B). Coadministration of Ac-DEVD-CHO with K1-5 significantly prevented endothelial cell apoptosis in microvessels induced by K1-5. These data provide convincing evidence that caspase-3 inhibitors prevent K1-5-induced microvessel apoptosis in tumors.

**Sequential Activation of Caspases-8, -9, and -3.** To gain additional insights with response to the activation of caspases, we measured the activity of caspases-8 and -9 at various time points. After K1-5 treatment, caspase-8 was highly activated within 3 h. This activation reached a maximal level at ~18 h and persisted for >24 h (Fig. 4A). Similarly, the maximal activation of caspase-3 was also detected ~18 h after exposure to K1-5 (Fig. 4B). In contrast, a delayed activation of caspase-9 was observed in K1-5-treated BCE cells (Fig. 4C). A significant proportion of caspase-9 was activated at ~18 h and reached a maximal level at ~24 h. These results suggest that activation of caspases-8 and -3 precedes caspase-9 activation. To further study the sequential events of caspase activation, we treated BCE cells with various caspase-specific inhibitors. As expected, z-IETD-fmk, a caspase-8-specific inhibitor, efficiently blocked caspases-8 and -9 activities (Fig. 4D). Similarly, z-LEHD-fmk, a caspase-9-specific inhibitor, completely blocked caspase-9 activity and partially attenuated caspase-8 activation. However, z-DEVD-fmk, a caspase-3 selective inhibitor, blocked caspase-9 activity ~100% and only slightly affected caspase-8 activation. These data suggest that the order of caspase activation in these cells is caspases-8, -3, -9, and -3, again. Like caspase-3 inhibitor, z-IETD-fmk and z-LEHD-fmk were also able to significantly prevent K1-5-induced BCE cell apoptosis (Fig. 4, E–H).

**K1-5 Binds to Endothelial Cell Surface ATP Synthase and Triggers Caspase Activation.** Although K1-5 was able to induce endothelial cell apoptosis by activation of intracellular caspases, this finding could not explain the specific inhibitory activity of K1-5 on endothelial but not other cells. To identify endothelial cell surface-specific molecules that could potentially mediate K1-5-induced apoptosis, we tested the possibility that K1-5 could bind to endothelial cell surface ATP synthase, identified as an angiostatin-binding molecule specifically expressed on endothelial cells (26, 27). We first demonstrated that BCE cells used in our system expressed both the α- and β-subunits of ATP synthase (Fig. 5, B and C). This interaction was specific because FITC-labeled antibody alone did not result in positive staining (Fig. 5A). A sensitive ELISA analysis demonstrated that K1-5 bound to purified, recombinant ATP synthase in a dose-dependent manner (Fig. 5D). We further demonstrated that K1-5 significantly inhibited F1 ATP synthase reverse activity (Fig. 5E). Having shown that K1-5 bound to endothelial cell surface ATP synthase, we performed studies to correlate this interaction with K1-5-induced endothelial apoptosis. Two specific neutralizing antibodies against the α- and β-subunits of ATP synthase were added to endothelial cells in the presence and absence of K1-5. Interestingly, both anti-ATP synthase antibodies sufficiently blocked K1-5-induced caspases-3, -8, and -9 activities (Fig. 5, F–H). Previously, it has been reported that angiostatin binds to endothelial cell surface integrin-αβ3 (38). To test whether integrin-αβ3 could play a role in mediation of K1-5-induced caspase activation and endothelial cell apoptosis, a neutralizing antibody against integrin-αβ3 was incubated with endothelial cells in the presence of K1-5. It appeared that the anti-integrin-αβ3-neutralizing antibody had only a little effect (~17%) on K1-5-induced caspase-3 activity, whereas it had no effect on K1-5-induced endothelial cell apoptosis (Fig. 5, I and J). These data demonstrate that interference of ATP synthase activation with antibodies blocks K1-5-induced endothelial cell apoptosis. Thus, endothelial cell surface ATP synthase plays a critical role in K1-5-induced caspase activation and endothelial cell apoptosis.

**DISCUSSION**

In the present study, we provide compelling evidence that K1-5-induced endothelial cell death contributes to its antiangiogenic mechanism. Since the discovery of angiostatin as the first endothelial cell-specific inhibitor produced in association with tumor growth (2), great efforts have been devoted to understanding the underlying mechanisms and signaling pathways. Despite these efforts, how this molecule specifically acts on endothelial cells remains an enigma. Although many interesting hypotheses have been raised to explain the specific effect of angiostatin on growing endothelial cells, these theories generally require additional experimental support. As both angiostatin and K1-5 are relatively large protein molecules, which cannot easily penetrate the cell membrane, it would be expected that...
the endothelial cell target molecules for angiostatin and K1-5 would be located on the cell surface. Therefore, several laboratories have focused on identifying endothelial cell surface molecules that bind to or interact with angiostatin. One intriguing study demonstrates that angiostatin, but not plasminogen, binds to ATP synthase on the surface of endothelial cells with its catalytic subunits located extracellularly (26). It appears that inhibiting this enzyme with a specific antibody impairs the antiproliferative effect of angiostatin (26). More recently, it was shown that angiostatin inhibits ATP synthase activity on endothelial cells (27). Another group used a genetic approach to identify an angiostatin-binding protein in endothelial cells (39). They have found that recombinant angiostatin expressed as a cytosolic protein interacts with angiostatin, an endothelial cell protein involved in motility (39). In addition to endothelial cell surface ATP synthase and angiostatin, integrin αvβ3 has been reported to bind to angiostatin (38). However, it has been difficult to study whether angiostatin or integrin αvβ3 transduces apoptotic signals triggered by angiostatin due to lack of specific neutralizing antibodies against bovine capillary endothelial angiostatin and other caspase components. Thus, it is important that further work should be devoted to develop these valuable reagents to know if these components other than endothelial cell surface ATP synthase also contribute to angiostatin/K1-5-induced endothelial apoptosis. However, our present findings suggest that it is unlikely that integrin αvβ3 plays significant roles in mediating K1-5-induced endothelial apoptosis. Furthermore, our present work explains, at least in part, the underlying mechanisms by which angiostatin/K1-5 specifically inhibits endothelial cell growth. If K1-5 binds to endothelial cell surface ATP synthase, it explains why K1-5 specifically acts on endothelial cells but not other cell types, because this endothelial cell surface molecule is only present on endothelial cells. Our findings that specific inhibition of various caspase activities completely impairs the antiproliferative, antiangiogenic, and antitumor effects of K1-5 suggest that caspase-mediated cellular apoptosis is essential for the angiostatic function of K1-5. Can we link K1-5-induced, caspase-mediated endothelial apoptotic pathways to its interaction with endothelial cell surface ATP synthase? The fact that antibodies against the α- and β-subunits of ATP synthase, known to block angiostatin-induced endothelial proliferation, can block K1-5-induced caspase activities clearly demonstrates that this cell surface ATP synthase is involved in triggering the intracellular caspase pathway. Our study on the cascade event of caspase activation indicates sequential activation of caspases-8, -3, and -9 (shown in schematic Fig. 6). Although the molecular details of these events deserve additional studies, it is clear that caspase-8 becomes activated first. The activation of caspase-8 by endothelial cell surface ATP synthase remains to be investigated. In addition to angiostatin and K1-5, several other endogenous angiogenesis, including endostatin, tumstatin, and thrombospondin-1, have been reported to induce caspase activation and endothelial cell apoptosis (18, 21–25). The relation between their endothelial cell surface receptors and induction of caspase activity/endothelial cell apoptosis remains to be further studied.

Angiostatin, endostatin, and several endogenous angiogenesis inhibitors, either alone or in combination with other therapeutic methods, have produced remarkable efficacy in suppression of primary and metastatic tumor growth in animal models (40–42). These promising animal studies have led to clinical trials of these inhibitors in suppression of human tumors. At the time of submitting this study, it still remains to be seen if these angiogenesis inhibitors are potent tumor suppressors in humans. Our present study provides important insight into the molecular mechanisms for understanding how these angiogenesis inhibitors act on newly formed blood vessels. Although several questions remain to be addressed, including the mechanisms behind angiogenesis inhibitors’ differential effect on active and quiescent endothelial cells and the roles of angiogenesis inhibitors in physiological and pathological angiogenesis, we believe that our work...
provides important clues for further clinical development of these angiogenesis inhibitors in cancer therapy.

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