Kinostatin, an Angiogenic Inhibitor, Inhibits Proliferation and Induces Apoptosis of Human Endothelial Cells

Yan-Lin Guo, Shujie Wang, Robert W. Colman

Abstract—We recently reported that domain 5 (D5) of high-molecular-weight kininogen inhibited critical steps required for angiogenesis. Thus, it was named kininostatin. To understand its mechanism of action, we further investigated the effects of D5 on basic fibroblast growth factor (bFGF)-induced endothelial cell proliferation and cell viability. We reported here that D5-inhibited cell proliferation of human endothelial cells stimulated by bFGF was associated with a significant reduction of cyclin D1 expression, which is a critical component required for the transition from G1 to S phase of the cell cycle. However, inhibition of cell proliferation by D5 was not due to an inhibition of extracellular signal–regulated protein kinase activity. Endothelial cells underwent apoptosis when cultured in a serum-free medium, which was prevented by bFGF. D5 reversed the protective effect of bFGF by 80%. Cells treated with D5 in the presence of bFGF showed typical morphological features of apoptosis, which was further confirmed by 2 additional assays: Hoechst 33258 cell staining and DNA fragmentation analysis. We conclude that the inhibition of endothelial cell proliferation and induction of apoptosis together represent a major contribution to the antiangiogenic activity of D5. (Arterioscler Thromb Vasc Biol. 2001;21:1427-1433.)

Key Words: kininostatin ◼ kinogen ◼ angiogenesis ◼ cell proliferation ◼ apoptosis

High-molecular-weight kininogen (HK) is a plasma protein that was first identified as a precursor of the bioactive peptide bradykinin, a potent vasodilatory agonist that regulates local blood pressure, microvessel permeability, and pain sensation. We now recognize that HK is a multifunctional protein that plays important roles in many pathophysiological processes, such as fibrinolysis, thrombosis, and inflammation.1,2 HK is a 120-kDa single-chain glycoprotein consisting of 6 domains (designated as D1 to D6 respectively) with each having distinct functions.2 After proteolytic cleavage by kallikrein and release of bradykinin contained within D4, cleaved HK (HKa) consists of a heavy chain containing D1, D2, and D3 and a light chain containing D5 and D6. The heavy and light chains are linked together by a single disulfide bond. The transition from HK to HKa involves major conformational changes3 and leads to a greater exposure of the D5 region. Compared with HK, HKa has an increased antiadhesive effect4 that is due to domain rearrangements.5 As a result, HKa acquires new properties. HK and HKa can specifically and reversibly bind to endothelial cells through D3 and D5 in a Zn2+–dependent manner. The endothelial cell is an important site for the generation of bradykinin and HKa, which, in turn, affect the physiology of endothelial cells. Although bradykinin has been intensively studied, the physiological implications of the generation of HKa are less clear. In a recent study,6 our laboratory reported that HKa and D5 exhibited a potent antiangiogenic effect by inhibiting important steps required for angiogenesis. The antiangiogenic activity of HKa and D5 was further demonstrated in an in vivo model by studying neovascularization in chicken chorioallantoic membranes, in which they markedly inhibited basic fibroblast growth factor (bFGF)-induced new blood vessel formation. Because D5 may function as an angiogenic inhibitor, we named it kininostatin.6

Angiogenesis is the process of formation of new blood vessels from existing blood tubes. It involves several steps, beginning with localized degradation of the basement membrane of the existing vessels by proteases bound to the endothelial cell membrane. This process is followed by the detachment of endothelial cells from adhesive proteins in the extracellular matrix and migration into the perivascular space, where endothelial cells proliferate rapidly. The new endothelial cells then form tubelike structures that eventually form new capillaries.7,8 This process is highly regulated by positive and negative effectors.9,10 Many growth factors and cytokines have been found to stimulate angiogenesis. bFGF and vascular endothelial growth factor (VEGF) are among the best-characterized angiogenic factors.9 Elevated levels of bFGF and VEGF have been found in the tumor milieu, in which angiogenesis is required for the supply of oxygen and nutrients for tumor growth.9 However, bFGF and VEGF are often detected in the tissue of healthy adults, in which the turnover of endothelial cells is very low with no apparent angiogenesis. The current hypothesis is that the maintenance of the quiescence of endothelium may be due to the presence of naturally occurring angiogenic inhibitors in...
normal tissues. Thus, the balance between the action of angiogenic stimulators and inhibitors will determine the dynamics of endothelial cell proliferation. The recent identification of several endogenous peptides with antiangiogenic activity provides important insight into how angiogenesis is negatively regulated. An emerging paradigm is that proteolytic fragments of plasma or extracellular matrix proangiogenic proteins are potent inhibitors of angiogenesis.\textsuperscript{10,11} Angiostatin (derived from plasminogen) and endostatin (a fragment of collagen XVIII) are prototypes of this group of polypeptides.\textsuperscript{12,13} Kininostatin is a new addition to this family of antiangiogenic peptides, whose generation from HK through proteolytic cleavage follows a model similar to that for the formation of angiostatin and endostatin.

The antiangiogenic effect of the documented angiogenesis inhibitors has been demonstrated in various experimental systems. They appear to interfere with one or more of the steps required for angiogenesis, such as cell proliferation, cell migration, and tube formation. However, the underlying mechanisms of their action at the molecular level remain unclear. Delineating their intracellular signaling pathways is a challenging task. Unlike bFGF and VEGF, which initiate a cascade of signaling events through their specific cell surface receptors, the antiangiogenic peptides originate from a variety of molecules, some of which have no known receptors and, thus, may use different mechanisms. To define their mode of action, detailed study must be devoted to each of these peptides. In the case of kininostatin, the signaling pathways that mediate its antiangiogenic activity have not been explored. In the present study, we have further investigated its effect on cell proliferation and have also provided evidence that kininostatin can also induce apoptosis of endothelial cells, which represents an important contribution to its antiangiogenic activity.

**Methods**

**Cell Culture**

Human dermal microvascular endothelial cells (HDMECs) and human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics. They were maintained in endothelial cell growth medium (EGM, containing growth factors and 10% FCS) at 37°C in a humidified incubator (5% CO\textsubscript{2}/95% air). HUVECs from 3 to 6 passages and HDMECs from 5 to 7 passages were used as stated in individual experiments. Cell culture dishes and glass coverslips were coated with 0.2% and 0.8% gelatin, respectively.

**Preparation of Recombinant D5 of HK and Cell Treatment**

Glutathione S-transferase (GST) and GST-D5 of HK recombinant protein (GST-D5) were prepared as previously described.\textsuperscript{6} Endotoxin levels in the preparations were determined with the chromogenic limulus amebocyte lysate assay by use of an endotoxin testing kit (BioWhittaker). The GST-D5 preparation (0.35 mg/mL) contains 14 endotoxin units (EU)/mL, whereas the GST preparation (0.31 mg/mL) contains 12 EU/mL. The final concentrations of endotoxin in the cell treatment media were 0.42 and 0.35 EU/mL for GST-D5 and GST, respectively. The endotoxin activity contained in the GST-D5 preparation can be completely neutralized by incubation with 5 \(\mu\)g/mL polymyxin B (Sigma Chemical Co) as analyzed by the method described by Ried et al.\textsuperscript{14} It has been determined that exogenous addition of endotoxin at a concentration of 0.50 EU/mL (\textasciitilde 0.05 ng/mL lipopolysaccharide; 1 EU/mL equals 0.1 ng/mL lipopolysaccharide) to the cell culture neither inhibits endothelial cell proliferation nor affects cell viability in a 48-hour incubation period. Thus, endotoxin contributed from recombinant protein preparation does not affect the effect of D5. This conclusion was further verified in an experiment in which 5 \(\mu\)g/mL polymyxin B was added to the cell culture medium to inhibit endotoxin activity. The effects of GST-D5 on cell viability and proliferation were not affected by the presence or absence of polymyxin B.

We have determined that GST-D5 has effects similar to those of HKa on in vivo tube formation\textsuperscript{9} and cell adhesion.\textsuperscript{15} Therefore, 300 nmol/L GST-D5 (simplified as D5 hereafter) was used for the present study. GST at the same concentration was used as a control. \(Zn^{2+}\) is required for HKa and D5 binding to endothelial cells; therefore, the cell culture medium contains 15 \(\mu\)mol/L \(ZnCl_2\) in the experiments in which cells were treated with D5.

**Cell Proliferation Analysis**

Cell proliferation was determined by bromodeoxyoxuridine (BrdU) incorporation analysis. Endothelial cells were plated onto glass coverslips in 6-well plates (1\( \times \)10\textsuperscript{4} cells per well, counted with a hemocytometer) and incubated in EGM for 3 hours, and then the medium was changed to medium 199 (serum-free medium). After incubation for 18 hours, the cells were treated with 10 ng/mL bFGF in the presence or absence of D5 for 30 hours. BrdU was added to the medium at the last 5 hours of incubation, and the cells then were fixed with 4% paraformaldehyde (with 0.5 mol/L \(ZnCl_2\)). BrdU incorporated into the DNA of proliferating cells was detected by the brown nuclei under a microscope according the method previously described.\textsuperscript{16}

**Immunocytochemical Analysis of Cyclin D1 Expression**

For analysis of cyclin D1 expression, endothelial cells were treated under the same conditions that were used for BrdU cell proliferation analysis. After the cells were stimulated with 10 ng/mL bFGF in the presence or absence of D5 for 24 hours, the cells were fixed with 4% paraformaldehyde in PBS (pH 7.4). The cells on the coverslips were incubated with anti–cyclin D1 antibodies (Santa Cruz Biotechnolgy). The cells expressing cyclin D1 were detected with an ABC immunocytochemical staining kit according to the manufacturer’s instructions (Vector Laboratories). The stained cells were analyzed and counted under a microscope.

**Cell Viability and Apoptosis Analysis**

Cell viability was assessed by a combination of morphological and metabolic changes after cell treatment by using the methods we have previously described,\textsuperscript{7,18} with some modification. The morphological changes of the cells were examined under a microscope periodically during and after cell treatment. Viable cells were quantified by neutral red uptake analysis. Briefly, cells were plated in 12-well dishes (5\( \times \)10\textsuperscript{4} cells per well) and incubated in EGM for 3 hours to allow for cell attachment. Cells were then treated with or without D5 in serum-free medium 199 in the presence or absence of bFGF for 48 hours. At the end of the cell treatment, the medium was removed, and the cells were incubated in DMEM containing 2% FCS and 0.01% neutral red for 90 minutes at 37°C. The uptake of the dye by viable cells was terminated by removal of the medium. The cells were washed briefly with 1 mL 4% paraformaldehyde. The internalized neutral red was extracted with 0.25 mL of a solution containing 50% ethanol and 1% glacial acetic acid. The absorbencies, which correlate with the amount of live cells, were determined at 560 nm.

Apoptotic cell death was analyzed by 2 methods: Hoechst 33258 (Hoechst) cell staining and DNA fragmentation. Hoechst cell staining was performed according to the procedures previously described.\textsuperscript{17} Briefly, endothelial cells were plated onto glass coverslips in 6-well plates (1\( \times \)10\textsuperscript{5} cells per well) and incubated in EGM for 4 hours to allow for cell attachment. The cells were then incubated with or without D5 in serum-free medium 199 containing 10 ng/mL bFGF for 48 hours. After treatment, the cells were fixed with 4% paraformaldehyde. Cells were washed with PBS, stained with 10 \(\mu\)mol/L Hoechst (Molecular Probes Inc) for 30 minutes, and analyzed under a fluorescence microscope with excitation at 340 nm.

DNA fragmentation analysis was carried out according to the procedures described by Sanchez-Alcazar et al.\textsuperscript{19} with some modification. The cells were treated in a manner similar to that used for...
Protein concentration was determined by using a protein assay kit. The cells were heated for 3 minutes at 90°C and then lysed by sonication at 4°C (8 pulses, output control 3) by using a Branson sonicator. The solution was centrifuged at 15 000g for 15 minutes. The supernatant was designated as whole-cell lysate and used for Western blot analysis. Protein concentration was determined by using a protein assay kit (Pierce).

**Cell Lysate Preparation**

After cell treatment, cells were washed twice with ice-cold PBS and scraped into cell-lysis buffer containing 10 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na3VO4, and 1% SDS. The cells were heated for 3 minutes at 90°C and then lysed by sonication at 4°C (8 pulses, output control 3) by using a Branson sonicator. The solution was centrifuged at 15 000g for 15 minutes. The supernatant was designated as whole-cell lysate and used for Western blot analysis. Protein concentration was determined by using a protein assay kit (Pierce).

**Western Blot Analysis**

Protein samples were subjected to SDS-PAGE, and the separated proteins were then transferred onto nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 and then incubated with anti-phospho-ERK (extracellular signal–regulated kinase) antibodies (New England Biolabs), followed by horseradish peroxidase–conjugated secondary antibodies according to the manufacturer’s instructions. The immunoblots were visualized by an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

**Results**

D5 Inhibits bFGF-Induced DNA Synthesis in Endothelial Cells

Our previous study showed that D5 inhibited bFGF-stimulated HUVEC proliferation. This conclusion is based on an assay that measures the total amount of DNA after cell lysis, in which the DNA content is proportional to the cell number.6 However, because the cells were incubated with D5 in a serum-free medium in the presence of bFGF for 48 hours, it is uncertain whether the decreased cell number/DNA content was due to the inhibition of mitosis and/or due to increased cell death in this extended incubation period. To clarify this question, we further analyzed the effect of D5 on bFGF-induced HUVEC proliferation at the single-cell level by using BrdU analysis. BrdU added to the medium will be incorporated into de novo synthesized DNA of proliferating cells. Immunostaining of BrdU with a peroxidase-conjugated antibody results in brown staining of the nuclei of proliferating cells. When only BrdU-positive cells were counted, D5 reduced 30% of the BrdU-positive cells stimulated by bFGF. GST as the control for GST-D5 had no effect (Figure 1, right; FGF+GST on graph). These results support the conclusion that D5 inhibited bFGF-stimulated proliferation of HUVECs, as previously determined by measurement of DNA content6; however, the reduced proliferating (BrdU-positive) cells can only partly account for the reduction of total cells observed in D5-treated samples. On careful examination of the D5-treated cells, we found that some BrdU-positive cells showed fragmented nuclei and shrinkage of cell bodies, a typical morphology of apoptotic cells (Figure 1, left; FGF+D5, indicated by the white arrow). These observations suggest that D5 is able to induce apoptosis and that apoptotic cell death also contributes to the reduction of BrdU cell labeling.

D5 Did Not Affect bFGF-Stimulated ERK Activation but Inhibits Cyclin D1 Expression

To delineate what steps of bFGF-stimulated cell proliferation were affected by D5, we tested the effects of D5 on 2 critical steps for cell cycle progression: activation of ERK and expression of cyclin D1. ERK is a subtype of mitogen-activated protein kinase and is stimulated by many growth factors and cytokines. Among the numerous cellular processes in which it is involved, ERK activation is considered to be an important early step in the initiation of mitosis.20 To test whether inhibition of cell proliferation by D5 was the result of the inhibition of bFGF-stimulated ERK, the cells were preincubated with D5 before stimulation with bFGF. ERK activation was determined by Western blot analysis with the use of antibodies that recognize only phosphorylated forms of ERK. Activation of ERK requires phosphorylation of a threonine plus a tyrosine residue in its active site. Therefore, phosphorylation of ERK on these 2 residues has often been used to indicate its activation by using antibodies specific for the phosphorylated form.21–23 Figure 2A shows the time course of bFGF-stimulated ERK activation, during which active ERK was detected as early as 5 minutes. Early activation of ERK is required for the activities of many key enzymes and transcription factors that ultimately regulate cell cycle progression.24 Surprisingly, neither short-term ERK activity (Figure 2B, 5 to 60 minutes) nor long-term ERK activity (Figure 3C, 24 hours) stimulated by bFGF was affected by D5 treatment. Phorbol myristate acetate (PMA) is known to stimulate ERK and was used as a positive control in this experiment. PMA strongly activated ERK, which was completely abolished by a MEK kinase inhibitor, PD098059 (PD in Figure 2D). MEK kinase is an upstream kinase of ERK.
Cyclins are positive cell cycle regulators, which activate cyclin-dependent kinases. Among several classes of cyclins required for completion of the cell cycle, cyclin D1 is required for cells to complete the G1 to S phase transition. The de novo synthesis of cyclin D1 is a signal of premitosis.25 As shown in Figure 3 (top), cells expressing cyclin D1 were detected by their brown nuclei after immunostaining with its specific antibodies. Consistent with the effect on cell proliferation (Figure 1), D5 significantly inhibited bFGF-stimulated cyclin D1 expression in HUVECs and HDMECs, with a more potent effect in HDMECs (Figure 3, bottom). The differences seen in HUVECs and HDMECs may be due to the cell-type difference in vitro, or it is also possible that HDMECs may be intrinsically more sensitive to angiogenic regulators than HUVECs inasmuch as HDMECs are likely to be affected by angiogenic regulators during angiogenesis. PMA strongly stimulated cyclin D1 expression in HUVECs, which was completely abolished by PD098059 (Figure 3), correlating with its inhibitory effect on ERK activity (Figure 2D). This result indicates that PD098059 inhibits PMA-stimulated cyclin D1 expression through inhibition of the ERK pathway in contrast to D5.

D5 Reduces Cell Viability and Induces Apoptosis of Endothelial Cells

The disruption of nuclei of endothelial cells treated with D5 (as indicated by the arrow in Figure 1, middle left) indicates that D5 may cause apoptosis. To confirm this observation, we first examined the effect of D5 on cell morphology by microscopic analysis. As illustrated in Figure 4 (top), many cells incubated in the absence of bFGF (serum and growth factor–free medium) for 48 hours lost their viability (CON in bFGF, FGF plus D5, bFGF plus 300 nmol/L D5, bFGF plus 300 nmol/L GST, bFGF plus 300 nmol/L GST, PMA, 1 µmol/L; and PMA+PD, 1 µmol/L PMA plus 10 µmol/L PD098059). Cyclin D1 was detected by immunostaining with anti–cyclin D1 antibodies. The cells expressing cyclin D1 (brown nuclei) were identified under a microscope with a ×40 objective lens. HUVECs are shown at the top. The graph at the bottom shows the percentage of cyclin D1–staining positive cells in HUVECs and HDMECs under the treatment conditions as indicated. At least 500 cells from >10 representative fields were counted. Results are mean±SEM of 3 experiments.

Figure 4. bFGF (FGF in Figure 4) prevented cell death. D5 significantly attenuated the bFGF protective effect (FGF+D5 in Figure 4), whereas the same concentration of GST did not have such an effect (FGF+GST in Figure 4). The dead cells showed typical morphological features of apoptosis, such as membrane blebbing and shrinkage of the cell body.

Viable cells under these experimental conditions were quantified by neutral red assay (Figure 4, bottom). In this assay, only viable cells can actively take up the neutral red dye. In the absence of bFGF (CON in Figure 4), neither D5 nor GST had an additional effect on the number of viable cells. Treatment of the cells with bFGF more than doubled the number of viable cells (FGF in Figure 4). D5 abolished the protective effect of bFGF (FGF+D5 in Figure 4) by 80%. HKa showed similar effects on cell viability and morphology of HUVECs (data not shown). These results indicate that D5 and HKa were able to induce apoptosis in addition to inhibition of cell proliferation.

Apoptotic cell death was further confirmed by 2 additional assays. Figure 5 shows Hoechst 33258 staining of HDMECs. Hoechst is a DNA binding dye that gives a bright blue color under a fluorescent microscope. The nuclei of viable cells are stained with Hoechst as blue round intact nuclei; apoptotic cells are detected as condensed/fragmented blue nuclei.
Consistent with the morphological features of apoptosis shown in Figure 4 (top), deprivation of growth factor (bFGF in Figure 5, top) caused disintegration of nuclei in many cells (as indicated by arrows), and this could be prevented by bFGF (FGF in Figure 5, top). Treatment of cells with D5 reversed the protective effect of bFGF (FGF + D5 in Figure 5, top). Approximately 20% of the cells showed apoptotic morphology (fragmented and condensed nuclei) among the cells treated with D5 in the presence of bFGF, similar to the apoptotic cells found in the cells incubated in the absence of bFGF (Figure 5, bottom). Similar results were obtained in HUVECs (data not shown). DNA fragmentation is a hallmark of apoptotic cell death. Figure 6 illustrates DNA fragmentation analysis of HUVECs that were treated in the same way as described in Figure 5. D5-treated cells in the presence of bFGF (lane 4) and cells cultured in the absence of bFGF (lane 5) showed a significant increase in the amount of degraded DNA.

HUVECs have been widely used in vitro experiments to study the steps involved in angiogenesis. Although, in many ways, they behave in a manner similar to that of human microvascular endothelial cells, it is recognized that HUVECs are derived from large vein vessels. It is necessary to verify the results obtained from HUVECs in microvascular endothelial cells, which are probably the type of endothelial cell involved in angiogenesis. It should be noted that D5 showed similar effects in HUVECs and HDMECs in the described experiments. Thus, HUVECs may be a reasonable model for in vitro studies.

**Discussion**

Successful angiogenesis not only depends on endothelial cell proliferation to provide new cells for tube formation but also critically relies on the preservation of cell viability. Inhibition of cell proliferation and induction of apoptosis of endothelial cells are 2 of the most effective ways to abolish angiogenesis, which may explain the effect of many inhibitors of angiogenesis. However, in many studies, inhibition of cell proliferation is often defined as the reduction of cell number caused by angiogenic inhibitors. The assays used for cell proliferation are based on either direct measurement of cell number or the amount of DNA as an indirect measurement of cell number. An increase in cell number or DNA content in the presence of angiogenic stimulators can reliably reflect an increased rate of mitosis. However, with a decreased cell number or a DNA level caused by angiogenic inhibitors, one cannot be certain whether this is due to the inhibition of mitosis and/or the induction of apoptosis. It is likely that the contribution by apoptosis to the reduced cell number could be significantly
of endothelial cells. No specific cell cycle proteins were identified as targets of angiostatin or kringle 5 in these studies. In the present report, D5 caused downregulation of the expression of cyclin D1, explaining, at least in part, its inhibitory effect on cell proliferation.

Virtually all known mitogens activate ERK, emphasizing the importance of ERK in the regulation of cell division. However, it is not obligatory to inhibit ERK to block the cell cycle. For example, the inhibition of cell proliferation by cAMP and ceramide are not via the modulation of ERK activity. Apparently D5-inhibited cyclin D1 expression was not due to the inhibition of ERK activity, because D5 did not affect bFGF-stimulated ERK activation. A similar result has been observed for angiostatin and canstatin. Canstatin, a novel angiogenic inhibitor derived from type IV collagen, inhibits endothelial cell proliferation without affecting growth factor–stimulated ERK activation. The authors speculated that canstatin did not primarily work by inhibiting proximal events activated by VEGF and bFGF receptors. The same explanation may apply to D5.

Another important finding of the present study is the observation that D5 was able to induce apoptosis. Although cell proliferation and apoptosis are 2 opposite physiological processes, accumulating evidence indicates that they are closely correlated events. In fact, apoptosis induced by some agents is primarily due to their ability to cause cell cycle arrest. For example, glucocorticoids induce apoptosis of proliferating cells by downregulating cyclin D3 and c-myc transcription factor. Previous studies of VEGF and bFGF have mainly focused on the signaling pathways that regulate their mitogenic effect. Recent findings suggest that they can protect cells from apoptosis by inducing synthesis of survival factors through signaling pathways independent of those used for mitogenesis. Blocking the expression of survival factors significantly reduces cell viability.

Although our results demonstrate that D5 can induce apoptosis and inhibit cell proliferation, it is not clear whether D5-induced apoptosis is interrelated with its ability to block cell cycle progression or whether the inhibition of cell proliferation by D5 is secondary to its proapoptotic effect. Nevertheless, we can speculate that D5 may cause a reduction in cell number by inhibiting the bFGF mitotic activity and/or by attenuating the antiapoptotic effect of bFGF independently. Alternatively, D5-induced apoptosis could be a result of an aborted mitosis; ie, it may initially cause an interruption in the cell cycle and result in cell cycle arrest. Consequently, an apoptotic pathway is activated to remove unsuccessful mitotic cells.

While the present article was in preparation, Zhang et al reported that HKa inhibits angiogenesis and induces apoptosis of endothelial cells. Their report confirms our previous study in which we showed that HKa and D5 exhibited a similar inhibitory effect on neovascularization. Thus, D5 retains the properties of HKa and is the primary domain responsible for inducing apoptosis and also for inhibiting cell proliferation. Zhang et al implied that the decreased cell number induced by HKa is primarily due to endothelial apoptosis, but the effect of HKa on the cell cycle was not analyzed. The present report provides evidence that the inhibition of endothelial cell proliferation by D5 correlates with a reduction of the expression of cyclin D1. Therefore, we conclude that the inhibition of endothelial cell proliferation and the induction of apoptosis represent a major contribution to the antiangiogenic activity of D5.
Acknowledgments

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References

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