Inhibition of Angiogenesis and Angiogenesis-dependent Tumor Growth by the Cryptic Kringle Fragments of Human Apolipoprotein(a) *

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Running title: Apolipoprotein(a) kringle domains inhibit angiogenesis and tumor growth
SUMMARY

Apolipoprotein(a) [apo(a)] contains tandemly repeated kringle domains that are closely related to plasminogen kringle 4, followed by a single kringle 5-like domain and inactive protease-like domains. Recently, the anti-angiogenic activities of apo(a) have been demonstrated both in vitro and in vivo. However, its effects on tumor angiogenesis and the underlying mechanisms involved have not been fully elucidated. To evaluate the anti-angiogenic and anti-tumor activities of the apo(a) kringle domains and to elucidate their mechanism of action, we expressed the last three kringle domains of apo(a), KIV-9, KIV-10, and KV, in Escherichia coli. The resultant recombinant protein, termed rhLK68, exhibited a dose-dependent inhibition of bFGF-stimulated human umbilical vein endothelial cell proliferation and migration in vitro, and inhibited the neovascularization in chick chorioallantoic membranes in vivo. The ability of rhLK68 to abrogate the activation of extracellular signal-regulated kinases appears to be responsible for rhLK68-mediated anti-angiogenesis. Furthermore, systemic administration of rhLK68 suppressed human lung (A549) and colon (HCT-15) tumor growth in nude mice. Immunohistochemical examination and in situ hybridization analysis of the tumors showed a significant decrease in the number of blood vessels and the reduced expression of VEGF, bFGF, and angiogenin, indicating that suppression of
angiogenesis may have played a significant role in the inhibition of tumor growth.

Collectively, these results suggest that a truncated apo(a), rhLK68, is a potent anti-angiogenic and anti-tumor molecule.
INTRODUCTION

Angiogenesis is a critical process involved in organogenesis during embryonic development, tissue regeneration during wound repair, and pathogenesis which occurs in diabetic retinopathy, rheumatoid arthritis, and tumor growth and metastasis (1-4). The onset of tumor angiogenesis can be triggered either by the up-regulation of tumor-released angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) or by the down-regulation of angiostatic factors such as thrombospondin, endostatin, and angiostatin (5). The reconstitution of angiostatic factors and/or the removal of angiogenic factors are thus promising plausible clinical strategies to treat rapidly overgrowing tissues such as tumors.

Numerous endogeneous angiogenic inhibitors have been identified, and several of them are currently being investigated in clinical trials for cancer therapies (6). One such molecule is angiostatin, which includes the first four kringle domains of plasminogen (7). When administered systemically, angiostatin significantly inhibits primary tumor growth as well as angiogenesis-dependent growth of metastases in mice and other pathogenesis involving corneal neovascularization (7-9). These anti-tumor effects are accompanied by a marked reduction of microvessel density within the tumor mass, indicating that suppression of angiogenesis may be associated with the inhibition
of tumor growth.

Lipoprotein(a) [Lp(a)] is a complex lipoprotein that consists of low-density lipoprotein (LDL) particles and apolipoprotein(a) [apo(a)], which is covalently attached to apoB-100 by a disulfide linkage (10). Apo(a) contains variable numbers of kringle domains that share 61-75% homology with kringle 4 of plasminogen (11). The kringle 4-like repeats of apo(a) are followed by a single copy of plasminogen kringle 5 (KV) and a protease region. The plasminogen kringle 4-like repeats of apo(a) (KIV) can be further classified into 10 different types, KIV-1 to KIV-10, on the basis of amino acid sequence (12). Each of these kringle domains, except KIV-2, is present in a single copy. The KIV-2 domain is present in differing numbers of identically repeated copies, which has resulted in a considerable size heterogeneity of Lp(a) in the human population.

Because of the high degree of sequence homology between plasminogen and apo(a), several studies have investigated the role of apo(a) in angiogenesis. Using the mouse sponge model and apo(a)-transgenic mice, Lou et al. observed that apo(a) had no effect on the regulation of angiogenesis (13). Based upon the results of a chick chorioallantoic membrane (CAM) assay, Ribatti et al. postulated that Lp(a) induces angiogenesis (14). However, Trieu and Uckun showed that LL/2 tumor growth was delayed with reduced microvessel density in apo(a)-transgenic mice, which suggests
that apo(a) can reduce angiogenesis in vivo (15). Furthermore, inspired by the discovery of the potent endogenous angiogenesis inhibitor angiostatin from the urine of tumor-bearing mice (7), Schulter et al. reported that full-size recombinant apo(a) and the naturally occurring urinary fragment of apo(a), which spans KIV-1 through KIV-4, inhibit in vitro tube formation of human microvessel endothelial cells in a fibrin matrix (16). Although urinary apo(a) fragments showed significant inhibitory effects, they were found to be much less effective than full-size apo(a). These results suggest that other structures in apo(a), such as the C-terminal kringle domains, are primarily responsible for its anti-angiogenic activity.

Among the kringle domains in the apo(a) protein, the last three appear to be critical for the structure and function of apo(a). KIV-9 possesses an additional cysteine residue that ensures covalent binding between apo(a) and apoB-100 (17). KIV-10 contains a high-affinity lysine binding site (LBS) that has been proposed to mediate the anchoring of Lp(a) or apo(a) to the vascular subendothelial matrix by binding to biological substrates such as fibrin and fibronectin (18-20). KV is the only apo(a) kringle domain that is homologous to plasminogen kringle 5. Based on these facts, we expressed these three kringle domains in Escherichia coli and determined the antiangiogenic and anti-tumor activities of the recombinant protein, henceforth referred to...
as rhLK68. In the present study, we show that rhLK68 inhibits endothelial cell proliferation and migration, possibly by blocking mitogen-activated protein kinase signaling in endothelial cells. We also found that rhLK68 can suppress angiogenesis-dependent tumor growth and down-regulate the expression of angiogenic factors within the tumor mass.
EXPERIMENTAL PROCEDURES

Construction of Plasmids

A cDNA encoding kringle domains KIV-9, KIV-10, and KV of human apo(a), termed LK68 (Fig. 1A), was amplified by PCR using a human liver cDNA library as the template with the oligonucleotide primers 5’-TCCATATGAAAAGCCCTGTGGTCCAGGAT-3’ and 5’-CGGGATCCTTAAGAGGATGCACA-3’, which contained linkers with *Nde* I and *Bam* H I restriction sites, respectively. The amplified 924-bp fragment, which spanned nucleotides 12,052 to 12,975 (see reference 10 for sequence), was digested with *Nde* I and *Bam* H I and cloned into pre-digested *E. coli* expression vector pET-11a (Novagen) to make pET11a/LK68. The DNA sequence of LK68 cloned into the pET11a/LK68 plasmid was confirmed using an ABI Prism 310 automated DNA sequencer (Applied Biosystems, Inc.).

Expression and Purification of Recombinant Human LK68 in *E. coli*

*E. coli* BL21(DE3) cells containing pET-11a/LK68 were grown in LB broth containing ampicillin (50 µg/ml) at 37°C with shaking. When the OD₆₀₀ of the culture reached 0.5, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. Cells were incubated for an additional 3 h and then harvested by centrifugation at 8,000 × g for 20 min at 4°C. The cells were disrupted by incubating with lysozyme (0.2
mg/ml) and DNase (2 µg/ml) in 20 mM Tris-HCl (pH 7.5) containing 0.2% Triton X-100 at room temperature for 30 min, and the cell lysate was centrifuged at 10,000 × g for 20 min. The isolated inclusion bodies were washed several times with 2% (w/v) sodium deoxycholate in Tris-HCl (pH 8.0) and solubilized in 7 M urea containing 100 mM β-mercaptoethanol. Refolding was accomplished in 20 mM Tris-HCl (pH 8.0) buffer in the presence of reduced and oxidized glutathione and L-lysine, and the solution was dialyzed against 20 mM sodium phosphate (pH 7.5). The refolded proteins were applied to a lysine-Sepharose 4B column, and the bound proteins were eluted with 0.2 M ε-aminocaproic acid (ε-ACA) in 20 mM sodium phosphate buffer (pH 7.5). Fractions containing LK68 were pooled, concentrated, and loaded onto a Sephadex G-25 column (2.5×20 cm) to remove ε-ACA. Chromatography with polymyxin-B beads (Sigma) was performed to eliminate any endotoxins. The bacterial endotoxin level was determined with the Limulus amebocyte lysate assay kit (Biowhittaker, Inc.). The purified proteins were dialyzed against PBS and stored at 4°C.

**Wound Migration Assay**

The ability of recombinant human LK68 (rhLK68) to block bFGF-stimulated human umbilical vein endothelial cell (HUVEC) migration was assayed in a monolayer denudation assay as described by Tang et al (21). HUVECs were maintained in 1.5%
gelatinized 24-well plates in EGM-2 (Clonetics) until confluency. Confluent endothelial
cells were wounded by scraping with a 2-200 µl pipette tip, which denuded a strip of the
monolayer that was 300 µm in diameter. Cultures were washed twice with PBS to
remove cellular debris. EBM media (Clonetics) supplemented with 1.0% fetal bovine
serum (FBS), 3 ng/ml bFGF, and rhLK68 proteins (0.001-1 µM) were added to the
monolayer and incubated at 37°C under 5% CO₂. Control cultures were incubated in
EBM plus 1.0% FBS without bFGF. The rate of wound closure was observed over an 8
h period. The cells that migrated into the denuded area were photographed with an
Olympus C-3030 digital camera and their numbers were counted.

**Endothelial Cell Proliferation Assay**

HUVECs were maintained in EGM-2 media. Approximately 3,500 cells were added to
each well of a 96-well tissue culture plate and incubated at 37°C in a 5% CO₂
atmosphere. After incubation for 24 h, the medium was replaced with EBM-2
containing 1% FBS and incubated for 18 h. After 30 min incubation with test samples,
bFGF was added to a final concentration of 3 ng/ml. Following 24 h of incubation, 0.4
µCi [³H]-thymidine (Amersham Biosciences, Inc.) were added to each well. Plates were
harvested after an additional 24-h incubation and thymidine incorporation was
measured using a scintillation counter. The experiments were performed in triplicate.
Analysis of MAPK Activity in Cultured HUVECs

The activities of three subtypes of mitogen-activated protein kinase (MAPK), including extracellular signal-regulated kinase (ERK), stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), and p38 MAPK, were analyzed by Western blotting of endothelial cell extracts with antibodies to their active and phosphorylated forms. HUVECs were cultured in EGM-2 until confluency and were then washed and grown in EBM-2 supplemented with 1% FBS. After 24 h, the medium was replaced with fresh low-serum medium with or without rhLK68. Occasionally, a specific inhibitor of MAPK kinase (MEK) U0126 (Cell Signaling Technology, Inc.) was added. Thirty min later, bFGF was added to 3 ng/ml. At various time points, the cells were washed with PBS and lysed with lysis buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1mM PMSF, and 1× protease inhibitor mixture]. Lysates were quantified for protein concentration and separated on 4-20% pre-casted SDS-PAGE gels. Western blots of control and rhLK68-treated lysates were performed to detect levels of active MAPK signaling molecules using anti-phospho-ERK, anti-phospho-JNK, anti-phospho-p38, or anti-phospho-MEK1/2 antibodies. To illustrate that equal amounts of total protein were loaded, the same blots were used to detect total protein using anti-ERK, anti-JNK, or anti-p38 antibodies. All of these
antibodies were purchased from Cell Signaling Technology, Inc.

**Chick Chorioallantoic Membrane (CAM) Assay**

The chick chorioallantoic membrane (CAM) appears in the yolk sac at 48 h, grows rapidly over the next 6-8 days, and stops growing on day 10. We incubated fertilized 3-day-old eggs at 37°C, and a window was made after extraction of ovalbumin. After 2 additional days of incubation, a Thermanox coverslip (Nunc, Inc.) containing rhLK68 protein was applied to the CAM of individual embryos. After 48 h, 20% fat emulsion was injected into the chorioallantois of the embryos, and the capillary formation around the Thermanox disc was examined. Data were presented as a percentage (%) of the number of CAMs showing inhibition of capillary formation out of the total number of CAMs tested.

**Tumor Studies in Mice**

Four-week-old female Balb/c nu/nu nude mice (Charles River, Japan) were housed in a sterile environment. Cages, bedding, food, and water were all autoclaved. The mice were maintained on a 12-h light/12-h dark cycle. Human lung cancer cells (cell line A549) or human colon cancer cells (cell line HCT-15) were purchased from the Korean Cell Line Bank (Seoul, Korea) and were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and antibiotics. Either $2 \times 10^7$ A549 or
HCT-15 cells were subcutaneously injected into the nude mice in the proximal midline of the dorsa. When tumors were palpable at day 7 after tumor implantation, mice were randomly divided into two groups. In the treatment group, rhLK68 (100 mg/kg body weight, daily) was administered subcutaneously. The control group was treated with PBS only. Treatment was continued for 12 (HCT-15) or 15 (A549) days, at which point all mice were sacrificed and the tumors were removed. The tumor size was measured every 2-3 days and the tumor volume was determined using the formula: width^2 × length × 0.52 as described (8).

**Histology and Immunohistochemistry**

Tumor specimens were dissected from mice and fixed in 10% buffered formalin solution overnight. They were then embedded in paraffin and sectioned in 4-μm thicknesses. The paraffin sections were de-paraffinized with xylene and were stained with hematoxylin and eosin (H&E), or treated with either monoclonal antibodies against human vascular endothelial growth factor (VEGF) (R&D Systems, Inc.) or α-smooth muscle actin (α-SMA) (Sigma), a monospecific goat antibody against angiogenin (R&D Systems, Inc.), or a monospecific rabbit antibody against von Willebrand factor (vWF) (DAKO, Denmark) by an indirect immunohistochemical method.

**RNA in situ Hybridization**
The cDNA sequences for vWF (498 bp), VEGF (573 bp), and bFGF (exon 3, 257 bp) were amplified by PCR using the following primers: 5’-CGGAAGTCCATGGTTCTGGATG-3’ and 5’-AAGTGTCTCAAAGTCCCGGATG-3’ for vWF; 5’-ATGAACCTTTCTGCTCTTTGG-3’ and 5’-TCACCGCCTTGCTTTGTACATC-3’ for VEGF; 5’-TTGTTTATCTCACTGTCTGTC-3’ and 5’-TCAGCTCTTTAGCAGACATTGG-3’ for bFGF. The resulting DNA fragments were subcloned into the pGEM-T Easy vector (Promega) by PCR-mediated unidirectional insertion (5’ end, Spe I; 3’ end, EcoR I). The plasmids were linearized by Spe I. Anti-sense probes labeled with digoxigenin-UTP were generated by T7 RNA polymerases (Boehringer Mannheim). Tumors were fixed in 4% paraformaldehyde solution and sectioned into 4-μm-thick RNase-free paraffin sections. After de-paraffinization, the sections were treated with proteinase K (10 μg/ml) for 15 min at room temperature, and endogenous alkaline phosphatase was inactivated by 0.2 N HCl. Hybridization in the tissue sections was performed at 50°C for 16 h under the same conditions as described by Lee et al (22). Detection of in situ hybridization was carried out using the Genius Detection System (Boehringer Mannheim), in which the specific transcripts were detected with an anti-digoxigenin antibody conjugated to alkaline phosphatase. The slides were washed several times with a solution containing 100 mM Tris-HCl (pH 9.5),
100 mM NaCl, and 50 mM MgCl₂, and then immersed in the color development solution [0.3 mg/ml nitroblue tetrazolium and 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M NaHCO₃ (Boehringer Mannheim)]. Color development was stopped by addition of 10 mM Tris-HCl (pH 8.0)/1 mM EDTA.

**Statistical Analysis**

The data are expressed as mean ± SE. *P* values were calculated from a student’s t-test. A value of *p*<0.05 was considered statistically significant.
RESULTS

Expression and Purification of Recombinant Human LK68 Protein

The cloned nucleotide sequence encoding LK68, which consists of human apo(a) kringle domains IV-9, IV-10, and V, is identical to that reported by McLean et al. (11), except for a single base change at position 12,605. A cytosine residue is present at this position instead of thymidine, which causes an amino acid change from Met to Thr. This substitution has also been reported by other groups (23-24) and appears to represent the predominant human apo(a) allele. Recombinant human LK68 (rhLK68) consists of 307 amino acids (Lys\(^{4003}\)-Ser\(^{4310}\)) from apo(a) plus a methione residue at its N-terminus. The calculated molecular mass and pI value of rhLK68 are 34,424 daltons and 6.13, respectively.

rhLK68 was highly expressed in E. coli BL21(DE3) as an inclusion body and accumulated to comprise about 20-30% of the total cellular protein (Fig. 1B). rhLK68 proteins were solubilized from inclusion bodies, refolded, and purified to homogeneity using lysine-Sepharose 4B affinity chromatography. The presence of a functional lysine-binding site in the purified rhLK68 indicates that rhLK68 was folded in the same way as the native apo(a) kringle domains. To ensure that refolded rhLK68 produced in E. coli retains its native conformation and maintains biological activity, an identical
cDNA representing rhLK68 was expressed in Chinese hamster ovary (CHO) cells and an endothelial cell migration assay in vitro and a CAM assay in vivo were performed. In contrast to E. coli-derived rhLK68, rhLK68 produced in CHO cells was highly glycosylated and secreted. Despite these differences, however, the anti-angiogenic activity of rhLK68 produced in E. coli was comparable to that of CHO-derived rhLK68, thereby confirming its correct folding and full biological activity (separate manuscript in preparation). To exclude any possible complications that may have been caused by contaminating bacterial endotoxins during purification, we used polymyxin B affinity chromatography to remove endotoxins from purified rhLK68 preparations. The endotoxin level was determined to be less than 5 EU/mg protein. The proteolyzed doses used for assays with endothelial cells showed no pyrogen-induced effects on the cells. Purified rhLK68 migrated as a single ~37-kDa band on SDS-PAGE under reducing conditions (Fig. 1C). The identity of purified rhLK68 was confirmed by N-terminal amino acid sequence analysis.

**Inhibition of Endothelial Cell Migration and Proliferation by rhLK68**

The effect of rhLK68 on the migration of endothelial cells was examined using a denudation injury model in confluent cell cultures. Confluent, scrape-wounded HUVEC monolayers (Fig. 2A) were incubated with bFGF in the presence or absence of rhLK68,
and the migration of HUVEC into the denuded area was observed over the following 8 h. HUVEC migrated into the wounded area in response to bFGF stimulation and covered up to ~70% of the wounded area (Fig. 2B and 2C). rhLK68 treatment inhibited bFGF-stimulated HUVEC migration in a dose-dependent manner with a range of 0.001-1 µM (Fig. 2D, 2E, and 2G). HUVEC migration was not affected by contaminating endotoxins in an amount similar to that contained in 1 µM rhLK68 (Fig. 2F). The concentration of rhLK68 required to inhibit the migration of HUVEC by 50% compared to controls (ED$_{50}$) was 230 nM (Fig. 2G).

We next tested the ability of rhLK68 to inhibit the proliferation of HUVECs stimulated by bFGF (3 ng/ml). rhLK68 also inhibited HUVEC proliferation in a dose-dependent manner with an ED$_{50}$ value of approximately 90 nM (Fig. 3). The inhibitory activity appears to be specific for endothelial cells, since rhLK68 failed to inhibit proliferation of non-endothelial cell lines such as CHO cells, mouse skin fibroblast cells (MSF), mouse embryonic fibroblast cells (NIH3T3), mouse adrenal tumor cells (Y1), mouse Lewis lung carcinoma cells, and a mouse embryonic liver/SV40 transformed cell line (TIB74) (data not shown). Moreover, rhLK68 appears to be non-cytotoxic to endothelial cells, since neither the morphology nor the adhesion properties of HUVECs were abnormally changed by the addition of rhLK68.
Inhibition of bFGF-induced ERK Phosphorylation by rhLK68

To study the possible molecular mechanisms involved in the anti-angiogenic activities of rhLK68, the effects of rhLK68 on bFGF-stimulated MAPK signaling in HUVECs was tested. Exogenous bFGF did not affect the activation of either SAPK/JNK or p38 MAPK (Fig. 4A). However, bFGF induced rapid phosphorylation of MAPK kinases (MEK1/2) and ERK1/2 in HUVECs (Fig. 4B). MEK1 and MEK2 were activated significantly within 5 min of bFGF treatment, while activation of ERK1 and ERK2 was observed at 5 min, reached a maximum value at 10 min, and then decreased to nearly background levels (Fig. 4B). These activation kinetic data are consistent with the fact that ERK1/2 is specifically activated by MEK1/2. Interestingly, treatment of HUVECs with rhLK68 selectively prevents the bFGF-stimulated phosphorylation of ERKs as early as 10 min after bFGF stimulation (Fig. 4B). These inhibitory effects were dose-dependent in a dose range of 0.1-1 µM (Fig. 4C), in which the migration and proliferation of HUVECs were significantly inhibited. The ability of rhLK68 to inhibit ERK activation appears to be specific to endothelial cells, as indicated by it’s inability to affect the PMA-stimulated activation of ERK in the THP-1 human monocytic leukemia cell line (data not shown). However, rhLK68 showed little effect on the activation of MEKs. To study the effects of ERK activation on endothelial cell
migration, ERK activation was selectively inhibited by treating cells with U0126, a specific inhibitor of MEKs. Pretreatment with U0126 blocked ERK1/2 phosphorylation in a dose-dependent manner (Fig. 4C) and significantly reduced the bFGF-induced migration of HUVECs (Fig. 4D) without affecting the adhesion and spreading properties of HUVECs. These results demonstrate that ERK activation is critical for the induction of endothelial cell migration and that rhLK68-mediated inhibition of endothelial cell migration may be achieved by interfering with the activation of ERKs. However, there was some discrepancy between the level of ERK dephosphorylation and the corresponding degree of migration inhibition. Although the levels of ERK dephosphorylation induced by 1 µM rhLK68 and 0.1 µM U0126 were similar, the resulting migration inhibitory activity was much higher in cells treated with 1 µM rhLK68. These results suggest that unlike U0126, which specifically inhibits ERK activation, rhLK68 may also affect other signaling pathway(s) involved in endothelial cell migration.

Inhibition of CAM Neovascularization

To determine its in vivo anti-angiogenic activity, the ability of rhLK68 to inhibit capillary development on the CAM, a widely-adopted in vivo model for studying angiogenesis, was assessed. rhLK68 inhibited the development of new embryonic blood
vessels without affecting the pre-existing vasculature (Fig. 5A), and there were no signs of toxicity in any of the chick embryos tested. rhLK68 significantly inhibited capillary growth in a dose-dependent manner at a dose range of 0.01-10 µg per CAM (Fig. 5B). When 10 µg rhLK68 were applied, an avascular zone around the disk was observed in 73.5% of the eggs, compared to 28.8% of the eggs that received PBS (Fig. 5B). These results indicate that rhLK68 can suppress neovascularization in vivo.

Suppression of Primary Tumor Growth by Systemic Administration of rhLK68

Angiogenesis is known to be activated during the early stages of tumor development (5), and angiogenesis inhibitors have a different degree of efficacy depending on the stage of carcinogenesis (25). Animal experiments were carried out to evaluate whether rhLK68 can suppress tumor growth prior to expansion of tumor. Two tumor cell lines, A549 (human lung carcinoma) and HCT-15 (human colon carcinoma), were implanted into the proximal midline of dorsa in nude mice. After 7 days, when tumors became palpable, tumor-bearing mice were subcutaneously injected with rhLK68 at a dose of 100 mg/kg/day. Such treatment with rhLK68 resulted in a significant suppression of primary tumors growth as shown in Fig. 6. The ratio of mean tumor volume of treated mice over control mice (T/C) was 0.247 (p<0.02) in A549 (Fig. 6A) and 0.403 (p<0.02) in HCT-15 primary tumors (Fig. 6B), respectively.

Decreased Expression of Angiogenic Factors in rhLK68-treated Tumor Tissues
To evaluate the consequences of rhLK68 treatment, implanted tumor tissues were examined by immunostaining and RNA in situ hybridization. Hematoxylin and eosin staining showed that HCT-15 cells from control mice were highly proliferative especially around the peripheral region of tumor and the cells were distributed in a fairly compact density and became somewhat less dense in tumor center (Fig. 7, a1 and a2). In contrast, tumor cells from rhLK68-treated mice were loosely arranged even in the periphery of tumor and frequently separated by collagenous fibrous tissues, with multiple necrotic spots in the tumor parenchyma (Fig. 7, b1 and b2). To examine the effect of rhLK68 on tumor neovascularization, immunostaining of vWF, an established endothelial cell marker, was performed (Fig. 7, c1 and c2). The close examination of entire region of tumor tissues (HCT-15) showed uneven distribution of vessels in both tumors from the control and the rhLK68-treated mice. In tumors from the control mice, vWF-positive cells were usually found in the actively proliferating peripheral region of tumor, whereas the middle of tumor parenchyma, where tumor cells are enlarged and less dense, showed scant distribution of vessels. On the contrary, tumor from the rhLK68-treated mice showed only a few vWF-positive cells even in the periphery of tumor tissues where cells are almost a half dense as the control tumor and appear to be necrotic state as well. The vWF-positive cells are almost non-detectable in the core
region of tumor from the treated mice. Estimation of vWF-positive cells in the 100 randomly selected fields at peripheral region of tumors from the control and the rhLK68-treated mice provided some measure of vascularity affected by rhLK68 treatment. Only a small fraction (15~20 %) of vWF-positive cells were remaining in tumors from the treated mice in comparison with the control (data not shown). The decreased expression of vWF was also confirmed at the transcriptional level by RNA in situ hybridization (Fig. 7, i1 and i2), which indicated that microvessel infiltrations were significantly reduced by rhLK68 treatment. Similarly, the results from immunostaining of α-SMA showed well-developed vascular structure in tumor tissues from control mice but sparse distribution of blood vessels in tumor tissues from rhLK68-treated mice (Fig. 7, d1 and d2). Since the implanted tumor cells are able to produce high levels of angiogenic factors such as angiogenin, bFGF, and VEGF, which switch on the angiogenic phenotype in the tumor implant, we next determined the effect of rhLK68 on the expression of these factors. Interestingly, the expression of angiogenin (Fig. 7, e1 and e2), VEGF (Fig. 7, f1 and f2 for protein, and h1 and h2 for mRNA), and bFGF (Fig. 7, g1 and g2) was dramatically decreased in rhLK68-treated tumor tissues compared to control mice as assessed by immunohistochemical and RNA in situ hybridization analyses. Consistent results were observed in implanted tumor tissues of A549 human
lung cancer cells (data not shown).
DISCUSSION

Angiogenesis is a complex, multi-step process that includes endothelial cell proliferation, migration and differentiation, degradation of the extracellular matrix, tube formation, and sprouting of new capillary branches. Since all of these steps could serve as potential targets for therapeutic intervention of angiogenesis, it is important to evaluate the effects of anti-angiogenic molecules on each individual step of the angiogenic process. In the present study, we first tested the effects of rhLK68 on the bFGF-stimulated proliferation and migration of endothelial cells. We found that rhLK68 specifically inhibits both endothelial cell proliferation and migration in a dose-dependent manner. These results indicate that rhLK68 may act directly on endothelial cells and affect multiple steps in the angiogenic process.

The mitogen activated protein kinase (MAPK) signaling pathway is a well-characterized signal transduction pathway that has been implicated in a wide range of cell biological events. In endothelial cells, at least three subtypes of MAP kinases are activated under different circumstances. Stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and p38 MAP kinase are activated in response to environmental stresses, while extracellular signal-regulated kinase 1 and 2 (ERK1/2) are activated by growth factors and are involved in cell proliferation and differentiation.
Among the MAPK signaling pathways, exogenous bFGF activates only ERK1/2, while it has no effect on the activation of SAPK/JNK or p38 in HUVECs. Activated ERKs regulate a variety of cellular functions that are critical to angiogenesis, e.g. stimulation of migration (26), tube formation (27), and expression of matrix metallopretease-9 (28) and urokinase (29). For this reason, we tested the hypothesis that rhLK68-mediated anti-angiogenesis is linked to attenuated ERK1/2 signaling in endothelial cells. In fact, in accordance with the results reported by Pintucci et al. (30, 31), migration of endothelial cells following wounding of endothelial cell monolayers appears to require ERK1/2 phosphorylation, since blocking of ERK1/2 activation by the specific MAPK kinase (MEK) inhibitor U0126 prevents bFGF-stimulated ERK activation and subsequently inhibits endothelial cell migration. In addition to endothelial cell migration, the ERK signaling pathway plays an important role in choroidal endothelial cell proliferation, although only about half of the total signaling appears to be mediated by this pathway (32). Therefore, our findings that rhLK68 abrogates bFGF-stimulated ERK1/2 activation strongly suggest that the inhibition of angiogenesis by rhLK68 may at least in part be achieved through the interference of ERK1/2 activation. The ability of endogeneous anti-angiogenic factors such as the 16-kDa fragment of prolactin (33), angiostatin (34), and plasminogen kringle 5 (35) to inhibit bFGF-induced ERK
phosphorylation in endothelial cells further supports the importance of this pathway and suggests an important mechanism for other inhibitors of angiogenesis.

The mechanism by which rhLK68 influences this intracellular signaling pathway is not all clear and remains to be further elucidated. Phosphorylation of many cell signaling molecules is regulated by two counteracting enzymes, protein kinases and protein phosphatases. Based on the finding that rhLK68 did not affect the bFGF-stimulated activation of MEKs, which are responsible for the phosphorylation of ERK1/2, protein phosphatases may play an important role in the inhibition of ERK1/2 activation by rhLK68. Our observations that sodium orthovanadate, an inhibitor of protein tyrosine phosphatase, compensates for both the rhLK68-induced dephosphorylation of ERKs and the decreased migration of HUVECs support this hypothesis (data not shown). Interaction with endothelial cell surface molecules such as integrin may also be a possible mechanism, since integrin is essential for sustained MAPK activation (36). Recently, Tuszynski et al. reported that angiostatin binds to annexin II (37), which is a profibrinolytic coreceptor for both plasminogen and tissue plasminogen activator on the surface of endothelial cells and facilitates the generation of plasmin. Competitive binding of angiostatin and plasminogen to annexin II may cause the reduced production of plasmin. Interestingly, a link between plasmin and the
activation of ERK was provided by Pendurthi et al. (38), who reported that plasmin is able to induce the activation of ERK1/2 through the protease-activated receptor 1 (PAR1). Similarly, apo(a) has also been reported to interfere with plasmin generation on endothelial cell surfaces by binding to annexin II (39). Binding of angiostatin and apo(a) to annexin II requires the lysine binding function of these molecules. In this context, the presence of a high affinity lysine binding site in KIV-10 suggests the possibility that rhLK68 may bind to annexin II and that this binding may lead to the dephosphorylation of ERK1/2 in a similar way as angiostatin.

In addition to the ERK signaling pathway, rhLK68 appears to affect other signaling pathway(s), since rhLK68 showed a more potent inhibitory activity of endothelial cell migration than U0126 even when the extent of ERK dephosphorylation by both molecules was equivalent. All of the signal transduction pathways that are involved in angiogenesis have not been fully elucidated. Therefore, more information is required to understand our observations in the full context of angiogenic signaling networks.

In addition to these in vitro anti-angiogenic activities, rhLK68 appears to be anti-angiogenic in vivo, as demonstrated by the suppression of neovascularization in the CAM assay.
Since tumor growth requires angiogenesis and rhLK68 inhibits angiogenesis both \textit{in vitro} and \textit{in vivo}, we evaluated the efficacy of rhLK68 as an inhibitor of angiogenesis-dependent tumor growth by using xenograft human tumor models in nude mice. Human lung and colon tumor growth in nude mice was suppressed by the systemic administration of rhLK68, resulting in 75.3\% and 59.7\% growth suppression \textit{in toto}, respectively. A variety of growth factors can stimulate angiogenesis, and bFGF and VEGF are the most commonly expressed in tumors. Tumor cells may overexpress one or more of these angiogenic factors, which may function synergistically in promoting tumor growth. Because of their critical roles in tumor-associated angiogenesis, bFGF and VEGF may be good targets for therapeutic intervention. Indeed, several studies have demonstrated that blocking the function of VEGF and its receptors can inhibit both tumor growth and metastasis. In this context, the suppression of angiogenesis-mediated tumor growth by rhLK68 appears to be the consequence of its ability to inhibit the expression of angiogenic factors such as angiogenin, bFGF, and VEGF in tumor tissues, which may in turn inhibit the capillary infiltration into tumors. Similar results have been reported by using other angiogenesis inhibitors. Joe \textit{et al.} demonstrated that angiostatin treatment induced almost complete suppression of bFGF and VEGF expression in brain glioma (40). Recently, Hajitou \textit{et al.} reported that
angiostatin and endostatin can down-regulate VEGF expression by both vascular cells and tumor cells (41), instead of acting exclusively on endothelial cells as initially believed. Although the reduced expression of angiogenic factors was clearly demonstrated in tumor tissues, it remains to be elucidated whether rhLK68 acts directly on tumor cells or endothelial cells and how rhLK68 exerts its regulatory function.

A number of fragments or cryptic domains of large proteins have been identified as angiogenesis inhibitors (42). Angiostatin and endostatin, proteolytic fragments of plasminogen and collagen type XVIII, respectively, the 16-kDa N-terminal fragment of prolactin, an N-terminally truncated platelet factor 4, and a C-terminal fragment of metalloprotease 2 named PEX, are potent angiogenesis inhibitors. Several noncollageneous domains from collagen type IV (canstatin, arrestin, and tumstatin) have also been shown to have anti-angiogenic activities. Likewise, it seems plausible that a cryptic fragment of human apo(a), LK68, can be categorized as this type of angiogenesis inhibitor, despite conflicting reports about the ineffectiveness of truncated apo(a) (15). Earlier studies showed that a truncated apo(a) protein with only six kringle 4 repeats from CHO cells neither delayed tumor growth nor impaired angiogenesis, while delaying of tumor growth and reduced angiogenesis were observed in apo(a)-transgenic mice expressing a recombinant apo(a) with 18 kringle 4 repeats (15).
Proteases produced from either tissues or tumor cells appear to largely contribute to the generation of endogenous angiogenesis inhibitors. In apo(a), protease cleavage sites for enzymes in the elastase and metalloproteinase families (43-45) have been identified in the linker between KIV-4 and KIV-5. Moreover, of the proteolytic fragments F1 (N-terminal) and F2 (C-terminal) generated by those proteases, only F1 was detected in human urine and plasma (46-47), partly because F2 can bind to matrix macromolecules such as fibrinogen or fibronectin, which may impede the excretion process. Based on these observations, it appears unlikely that LK68 fragments are generated in vivo.

In conclusion, we have demonstrated that a recombinant kringle fragment derived from human apolipoprotein(a), called rhLK68, inhibits angiogenesis both in vitro and in vivo. rhLK68 inhibits endothelial cell migration and proliferation, and these effects may be achieved in part through the interference of the bFGF-stimulated MAPK signaling pathway in endothelial cells. In association with its ability to down-regulate the expression of angiogenic factors in tumors, rhLK68 can suppress solid tumor growth. Collectively, these anti-angiogenic and anti-tumor activities of rhLK68 suggest that, though not an endogenous angiogenesis inhibitor per se, rhLK68 may have the potential to be a useful inhibitor of a number of angiogenesis-dependent diseases including
cancer.

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FOOTNOTES

1The abbreviations used are: VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; Lp(a), lipoprotein(a); Apo(a), apolipoprotein(a); HUVECs, human umbilical vein endothelial cells; MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; CAM, chick chorioallantoic membrane; vWF, von Willebrand factor; α-SMA, α-smooth muscle actin.
Fig. 1. **Expression and purification of rhLK68 protein.** A, schematic representation of apo(a), which contains 10 unique kringle 4-like domains (designated IV-1 through IV-10; kringle IV-2 is variably repeated), one kringle 5-like domain (KV), and a protease domain, aligned with LK68. B, SDS-PAGE of rhLK68 expressed as an inclusion body: lane 1, uninduced bacterial lysate; lane 2, IPTG-induced bacterial lysate; lanes 3 and 4, soluble and insoluble fractions of IPTG-induced bacterial lysate, respectively. C, SDS-PAGE of purified rhLK68. rhLK68 proteins are indicated by arrows. Molecular mass markers (in kilodaltons) are shown on the left.

Fig. 2. **Effects of rhLK68 on the migration of HUVECs.** Photomicrographs showing cells after initial scraping (panel A) and migration of cells after 8 h in the absence (panel B) or presence (panel C) of 3 ng/ml bFGF. Treatment of rhLK68 proteins, 10 nM or 1 µM (panels D and E, respectively), inhibited the migration of HUVECs in a dose-dependent manner, but matched endotoxin did not affect HUVEC migration (panel F). Dotted lines indicate the area occupied by the initial wound. G, graphical representation of cell migration. Data are expressed as a percentage of the number of migrated cells in control cells treated with bFGF only (mean ± SE). The data are representative of three independent experiments. *, p<0.0001; **, p<0.0000001; ***, p<0.000002 versus bFGF-treated control.
Fig. 3. Inhibition of bFGF-stimulated HUVEC proliferation by rhLK68. HUVECs were cultured with bFGF (3 ng/ml) and treated with concentrations of rhLK68 ranging from 1-1,000 nM for 24 h, followed by incubation with [3H]-thymidine as described in “Experimental Procedures”. Each experiment was performed in triplicate. The data are representative of three independent experiments. Graph bars represent mean cpm ± SE. *, p<0.05; **, p<0.02 versus bFGF-treated control cells.

Fig. 4. Effects of rhLK68 on bFGF-induced ERK1/2 activation in HUVECs. A, confluent HUVECs were cultured in EBM-2 plus 1% FBS for 24 h and stimulated with bFGF (3 ng/ml) for the time periods shown. Cells were lysed and subjected to SDS-PAGE and Western blotting analyses for phospho-ERK1/2, phospho-SAPK/JNK, and p38 MAPK. B, HUVECs were preincubated with or without rhLK68 (1 µM) for 30 min and then stimulated with bFGF (3 ng/ml). At the indicated time after bFGF stimulation, cell lysates were analyzed by Western blotting for the phosphorylation of MEK1/2 and ERK1/2. The histograms represent the relative phosphorylation of MEKs or ERKs in the absence (open bars) or presence (filled bars) of rhLK68. Data are means ± SE of three independent experiments. C, HUVECs were preincubated with either rhLK68 (0.1 or 1 µM) or U0126 (0.1, 0.5, or 1 µM) for 30 min and stimulated with bFGF (3 ng/ml) for 30 min. ERK1/2 phosphorylation was analyzed by Western blotting. The histograms represent the relative intensity of phosphorylated ERK1/2 as determined by densitometric analyses. Results shown are the means
of three independent experiments (± SE). D, bFGF-stimulated HUVEC migration in the presence of either rhLK68 or U0126. Wound-associated migration of HUVECs was analyzed as described in “Experimental Procedures”. Data are expressed as the percentage of the number of migrated cells in bFGF-stimulated control cells (mean ± SE) from a single experiment representative of three.

Fig. 5. CAM assay showing inhibition of angiogenesis in vivo. Thermanox coverslips containing a range of concentrations of rhLK68 were applied to the CAMs of 3-day-old chick embryos. After 48 h, the formation of avascular zones (≥ 5 mm in diameter) was analyzed. A, photographs of a representative CAM applied with a Thermanox plate containing PBS (left) or rhLK68 (right). Dotted circles indicate the Thermanox plates. B, dose-dependent inhibition of angiogenesis in the CAM by rhLK68. Inhibition (%) of treated samples was measured as the number of CAMs showing an avascular zone divided by the total number of treated CAMs. Values are means ± SE of three independent experiments.

Fig. 6. Suppression of primary tumor growth by rhLK68. Human lung carcinoma (A549) (panel A) and human colon carcinoma (HCT-15) cells (panel B) were subcutaneously implanted into nude mice, and rhLK68 (100 mg/kg/day) or PBS was injected daily via subcutaneous routes for 12-15 days starting from the 7th day after tumor implantation (n=5/group). Tumor volume was calculated by the formula width^2
× length × 0.52. *, p<0.02. Each value represents the mean ± SE.

Fig. 7. **Immunohistochemical observations and RNA in situ hybridization analysis of tumor tissues.** Hematoxylin and eosin (H&E) staining of tumor tissues from control (a1 and a2) and rhLK68-treated mice (b1 and b2) implanted with human colon carcinoma (HCT-15) cells. Magnifications, ×100 (a1 and b1) and ×400 (a2 and b2). Immunostaining of tumor tissues using an antibody against vWF (c1 and c2), α-SMA (d1 and d2), angiogenin (e1 and e2), and VEGF (f1 and f2). RNA in situ hybridization analysis showing the mRNA expression of bFGF (g1 and g2), VEGF (h1 and h2), and vWF (i1 and i2). Each panel comprises two photomicrographs that represent tumor tissues from control (c1-i1) and rhLK68-treated (c2-i2) mice under the same magnification, ×400. Control RNA in situ hybridization of tumor tissues using sense-strand probes corresponding to bFGF (j1), VEGF (j2), and vWF (j3).
FIG. 2

A B C

D E F

G

% Migrated Cells

rhLK68 concentration (nM)
FIG. 4A

A

| (kDa) | 0  | 2  | 5  | 10 | 30 (min) |
|-------|----|----|----|----|----------|
| 50    |    |    |    |    | p-ERK1/2 |
| 37    |    |    |    |    | ERK1/2   |
| 50    |    |    |    |    | p-JNK    |
| 50    |    |    |    |    | p-p38    |
| 37    |    |    |    |    | p38      |
FIG. 4D

- % Migrated cells
- Sample treatment:
  - U0126 (μM): 1, 0.1, 0.5, 1
  - rhLK68 (μM): 3
  - bFGF (ng/ml): 3
FIG. 5

A

Control  

rhLK68 treatment

B

% Inhibition

rhLK68 (μg/CAM)

p<0.000002

p<0.001

p<0.01
FIG. 6

A

B

![Graph A](image1)

![Graph B](image2)
Inhibition of angiogenesis and angiogenesis-dependent tumor growth by the cryptic Kringle fragments of human apolipoprotein(a)

Jang-Seong Kim, Ji-Hoon Chang, Hyun-Kyung Yu, Jin-Hyung Ahn, Jung-Sun Yum, Suk-Keun Lee, Kyung-Hwan Jung, Doo-Hong Park, Yeup Yoon, Si-Myung Byun and Soo-II Chung

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