The recombinant kringle domain of urokinase plasminogen activator inhibits *in vivo* malignant glioma growth

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In a previous report, the recombinant kringle domain (UK1) of the urokinase type plasminogen activator (uPA) showed antiangiogenic activity. Here, we investigated *in vivo* antitumor effects of the UK1 of human uPA employing a brain tumor model. The systemic administration of UK1 purified from pichia expression (10 and 50 mg/kg/day intraperitoneally for 25 days) led to suppress the growth of a U87 human glioma xenograft, implanted into the brains of male BALB/cSlc nude mice, by 35% and 80%, respectively. In the immunohistochemical analysis, the tumors treated with UK1 showed decreased vascularity and expression of angiogenesis-related factors including vascular endothelial growth factor (VEGF), angiogenin, α-smooth muscle actin, von Willebrand's factor, and CD31 (PECAM-1 [Platelet endothelial cell adhesion molecule-1]), and increased apoptosis. UKl inhibited the *in vitro* proliferation and tube formation of VEGF-stimulated endothelial cells but not the proliferation of glioma cells. These results suggest that UK1 inhibits the malignant glioma growth by suppression of angiogenesis. (*Cancer Sci* 2007; 98: 253–258)

Malignant gliomas, the most common primary brain tumors, are extremely aggressive and highly angiogenic tumors with a very poor prognosis despite available intensive therapies including surgery, irradiation and chemotherapy.[1,2] Angiogenesis is an important feature associated with malignant glioma growth and progression.[3] The blood–brain barrier or blood–tumor barrier, which hinders drug delivery to brain tumors, is considered to be one of the main problems associated with systemic chemotherapy of brain tumors.[1,4] Angiogenesis inhibition, targeting endothelial cells rather than tumor cells, may especially be suitable for the treatment of malignant gliomas. Several antiangiogenic therapies have shown antitumor effects only in the preclinical setting but not in clinical studies.[5,6] It is important to investigate a new antiangiogenic molecule and its action mechanism for the development of antiangiogenic tumor therapy.

Urokinase-type plasminogen activator (uPA) belonging to a protein family that contains a kringle domain plays an important role in inflammation, invasion, angiogenesis, and tumor metastasis by pericellular plasminogen activation.[7–9] uPA is a multidomain protein composed of a carboxyl-terminal protease domain and an amino-terminal fragment (ATF) which can be further subdivided into a growth factor-like domain (aa 4–43) and a kringle domain (aa 45–135, UK1). uPA is highly expressed in malignant brain tumors;[10] its binding to the uPA receptor (uPAR) is enhanced by tumor progression and invasion by proteolysis of the extracellular matrix.[11] The ATF fragment of uPA has been reported to inhibit glioma growth and invasion properties *in vivo*.[12] Interestingly, in our previous study, we demonstrated that the kringle domain (Asp^{45} – Lys^{135}), a subdomain of ATF, possesses an antiangiogenic activity, possibly with a different mechanism of ATF.[13,14] Thus, it warrants further investigation for its antitumor effect and action mechanisms.

Here, we report that systemic administration of UK1 efficiently suppressed *in vivo* malignant glioma growth in nude mice, and reduced the expression of angiogenesis-related factors in tumor tissues.

Materials and Methods

Cell culture. Human malignant glioma cell lines U87, A172 and U373 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). U87 was maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY) medium supplemented with 10% fetal bovine serum (FBS) and incubated in a humidified incubator at 37°C with 5% CO_2. Human umbilical vein endothelial cells (HUVECs) were maintained as described previously.[13]

Expression and purification of the recombinant UK1. The recombinant UK1 expressed in *Pichia pastoris* was purified as described previously.[15]

Endothelial and glioma cell proliferation assay. For HUVECs, [^{3}H] thymidine incorporation assay was performed as follows: 2×10^4 cells/well were plated on gelatinized 24-well culture plates and incubated in M199 containing 10% FBS, 90 μg/mL heparin, and 1% antibiotics for 24 h. The medium was replaced with 0.25 mL M199 with 5% FBS, 90 μg/mL heparin, and 1% antibiotics, and the test sample was applied. After 30 min incubation, 0.25 mL M199, 5% FBS, 90 μg/mL heparin, 1% antibiotics, and a growth factor (10 ng/mL vascular endothelial growth factor [VEGF]) was added. After 18 h, 1 μCi (0.037 MBq) [^{3}H]thymidine (Dupont NEN, Boston, MA) was added to each well. After further incubation for 6 h, cells were fixed with methanol, washed with cold 10% Trichloroacetic acid (TCA) solution three times, and dissolved in 0.25 N NaOH with 1% Sodium dodecyl sulphate (SDS). Radioactivity was determined using a liquid scintillation counter. Each experiment was performed in triplicate. For nonendothelial cells, [^{3}H] thymidine incorporation assay was performed the same as above, having only difference with DMEM media.

Endothelial cell morphogenesis (Matrigel tube formation) assay. Each well of a prechilled 24-well cell culture plate was coated with 300 μL of Matrigel (Becton Dickinson, Heidelberg, Germany) and incubated at 37°C for 30–45 min. HUVECs were harvested with trypsin and 4×10^4 cells were resuspended in 300 μL media with 10% FBS. M199 medium supplemented with VEGF

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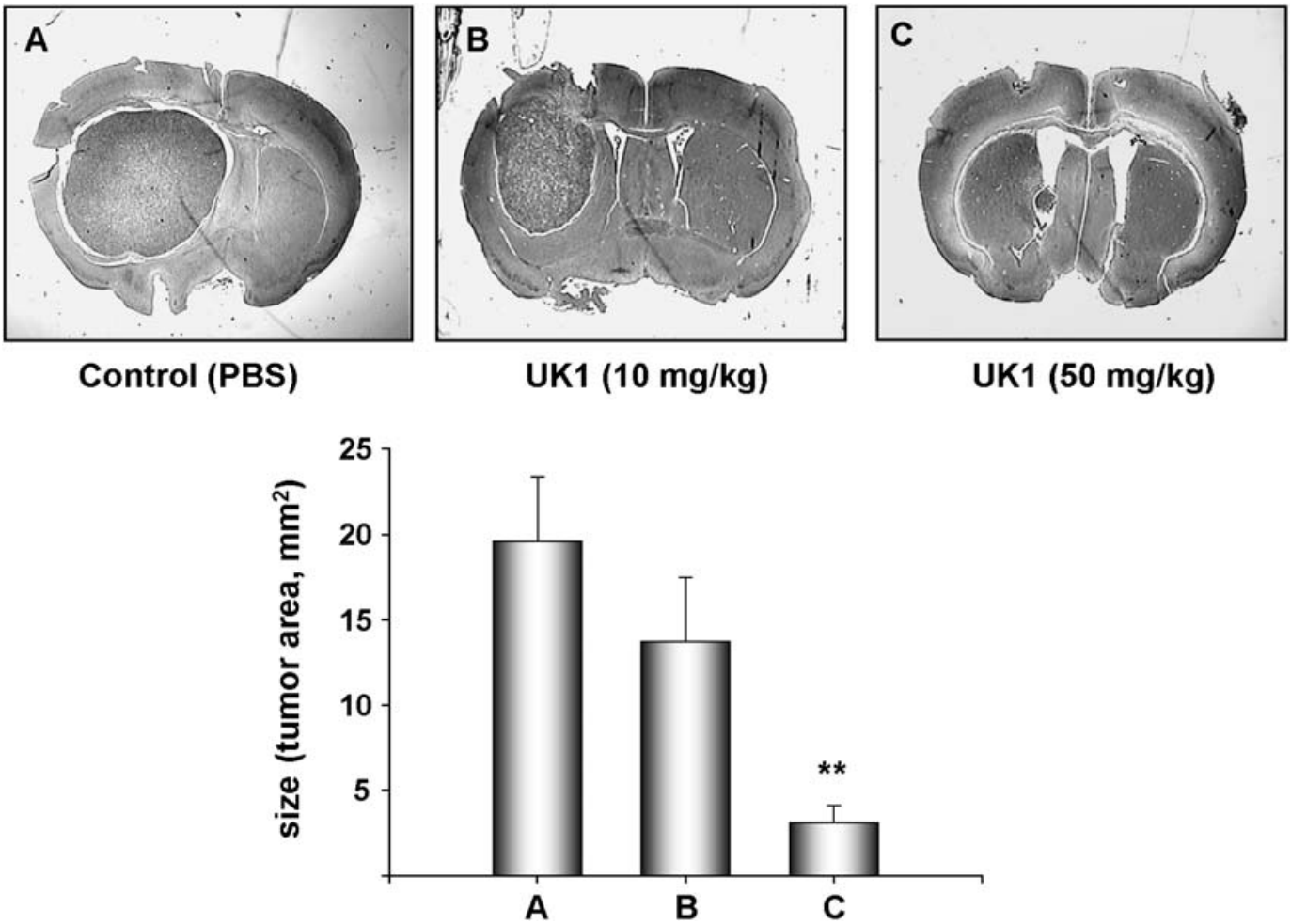

Fig. 1. Growth inhibition of brain tumors by recombinant kringle domain (UK1) treatment. Representative brain tumors of controls (A) and animals treated with UK1 10 mg/kg (B) and 50 mg/kg (C) stained with hematoxylin and eosin. The tumor sizes were determined by the measurement of a frontal section area at the maximal brain tumor dimension. $^{**}P < 0.001$ versus control.

(10 ng/mL) and UK1 (0.32 and 0.64 μM) was incubated with cells before plating onto the Matrigel-coated plate. After 8 h of incubation, on the Matrigel at 37°C under 5% CO_2, the medium was aspirated and tube formation was observed through an inverted microscope.

Brain tumor animal models treated with UK1. Brain tumor animal models were made as described previously.[16] There were 2×10^5 U87 human glioma cells in a volume of 3 μL PBS, which were injected slowly into the brain with a Hamilton syringe. Three days after the intracerebral (i.c.) inoculation of the U87 glioma cells, the animals were treated with daily intraperitoneal (i.p) administration of phasphate buffered saline (PBS) (control group, $n = 7$), or UK1 (10 mg/kg and 50 mg/kg, experimental group, $n = 7$) for 25 days. Two days after the final treatment, the animals were sacrificed and fixed using intracardiac perfusion of 4% paraformaldehyde in PBS, and the brains were removed. Serial dissections on the frontal plane, in 1 mm thickness, were performed using a knife blade. Among the serial sections, the median cut surface was selected as a representative size of the tumor; each median section was digitalized for area analysis by Image Pro-4.0 (Media Cybernetics, Silver Spring, MD).

Immunohistochemical analysis. Tumors were dissected from sacrificed animals, fixed in 10% buffered formalin solution, and then embedded in paraffin according to standard histological procedures. Immunohistochemical stains were applied using monoclonal antibodies against proliferating cell nuclear antigen (PCNA; DAKO, Carpinteria, CA), von Willebrand's factor (vWF; R & D Systems, Inc., Minneapolis, MN), α-smooth muscle actin (α-SMA; Sigma, St. Louis, MO), angiogenin (R & D Systems, Inc., Minneapolis, MN), hypoxia inducible factor 1α (HIF-1α NOVUS Biological, Littleton, CO), caspase 9 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and VEGF (R & D Systems, Inc). Sections were sequentially incubated with biotin-labeled secondary antibodies and streptavidin-peroxidase complex (DAKO), and then visualized by diaminobenzidine (DAB) colorization (DiNonA, Seoul, Korea). In order to compare the angiogenesis level in the microsections, the digitalized microphotographs were analyzed using Image Pro-4.0 (Media Cybernetics, Silver Spring, MD) program to define the vessel area per 0.12 mm^2 of tissue section, and to calculate them into percentage (%). Twenty representative microphotographs from each experimental group were used for this statistical analysis.

To detect apoptotic cells in brain tissue sections, the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) method (ApopTag *in situ* apoptosis detection kit; Oncor, Gaithersburg, MD) was used according to the manufacturer's instructions.

Statistical analysis. Student's *t*-test was used to determine significance. The data were expressed as the means ± SE. A value of $P < 0.05$ was statistically significant.

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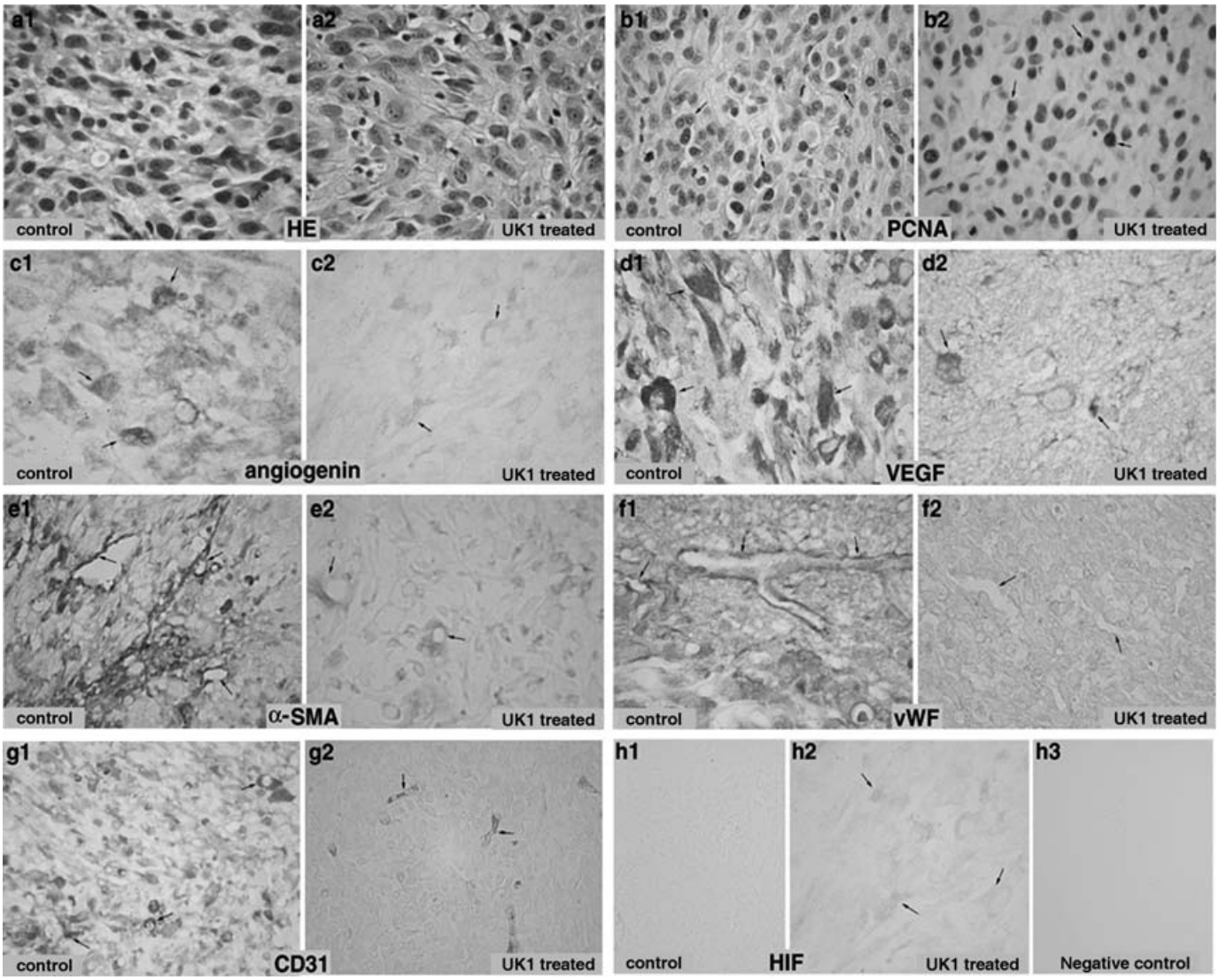

Fig. 2. Histologic examination of the brain tumors. Brain tumor tissues from the animals treated with intraperitoneal administration of phosphate buffered solution (left panel, a1–h1) or recombinant kringle domain (UK1) (50 mg/kg) (right panel, a2–h2) were used for hematoxylin and eosin (H&E) staining, immunohistochemistry for proliferating cell nuclear antigen (b1, b2), angiogenin (c1, c2), vascular endothelial growth factor (d1, d2), α-smooth muscle actin (e1, e2), vWF (f1, f2), CD31 (g1, g2) and hypoxia inducible factor 1α (h1–h3) (×400 magnification).

Results

Suppression of *in vivo* U-87 glioma growth by UK1. To evaluate the suppression of brain tumor growth by antiangiogenic inhibitor, we addressed the *in vivo* antitumor effect of UK1 on intracerebral U-87 human glioma xenograft model in nude mice. After tumor cell inoculation, tumors were allowed to grow until established with minimal tumor burden, and then mice were randomized and were treated. As shown in Figure 1, the control group showed steady intracerebral tumor growth up to 18.3 ± 5.79 mm^2 in the median area. On the other hand, the animals treated with UK1 showed a decreased average median area of tumor sections, 11.66 ± 3.08 mm^2 (35% decrease versus control, 10 mg/kg, $P < 0.3415$) and 3.63 ± 1.60 mm^2 (80% decrease versus control, 50 mg/kg, $P < 0.0001$). No signs of toxicity, as measured by weight loss and an external appearance, were observed in any of the mice treated with UK1.

Effect of UK1 on *in vivo* angiogenesis, proliferation and apoptosis. The effects of UK1 on *in vivo* proliferation, angiogenesis, and apoptosis were assessed by immunohistochemistry of brain tumor tissues. The tumor tissues of UK1-treated mice showed loosely arranged tumor cells which were rather cuboidal to polygonal in shape, while the tumor tissue of control mice showed condensed tumor cells which were almost spindle in shape by hematoxylin and eosin stains (Fig. 2a1,a2). The tumors of both control and experimental groups showed a strong reaction to PCNA (Fig. 2b1,b2), which was not affected by UK1 treatment on tumor cell proliferation rate (Fig. 3b). The tumor treated with UK1 showed a significantly decreased immunoreaction to angiogenin, VEGF, α-SMA, vWF, and CD31, of which reactions were strong in the tumor cells of control group (Fig. 2c–g). On the other hands, the tumor cells treated with UK1 showed greater increased reaction of HIF1 than the tumor cells of the control group. Immunostaining for vWF and α-SMA clearly showed reduced vessel density in the parenchymal tumor tissue in UK1 treated mice compared to controls. These immunoreactions were identical with analysis of vessel area, showing that the UK1-treated tumor greater vessel area percentage decrease ($2.87 \pm 0.688\%$) than the control tumor ($6.09 \pm 0.857\%$) (Fig. 3b). The immunoreaction of caspase-9 was significantly

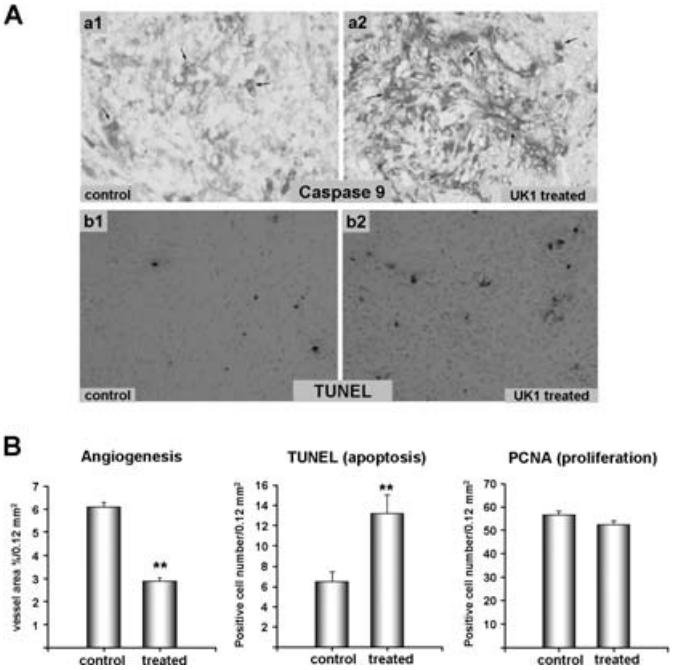

Fig. 3. Histologic examination of apoptosis and analysis of angiogenesis, apoptosis, and proliferation of U87 glioma from control and recombinant kringle domain (UK1)-treated mice. (A) Photomicrographs of stained tumor tissue from control (phosphate buffered solution) (left panel) and UK1 (50 mg/kg) treated mice (right panel) were used for caspase 9 (a1, a2) and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling assay (b1, b2) (×400 magnification). (B) Microvessel area was measured per 0.12 mm^2 of tissue section, and was calculated into percentage (%). Apoptosis was measured using Terminal Deoxynucleotidly Transferase (TdT) labeling to identify the number of cells with fragmented DNA per 0.12 mm^2 of tissue section. Cell proliferation was determined as the number of proliferating cell nuclear antigen-positive cells per 0.12 mm^2 of tissue section. $^{**}P < 0.00001$. Representative data are shown. TUNEL, transferase-mediated dUTP-biotin nick end-labeling; PCNA, proliferating cell nuclear antigen.

increased in the UK1 treated group (Fig. 3a) and the TUNEL assay revealed a 2.2-fold increase of apoptotic cells (13.2 ± 1.8 versus 6.0 ± 0.97, respectively, $P < 0.0002$) in the tumors treated with UK1 compared to the control group (Fig. 3b). These findings are consistent with the results of previous studies showing that inhibition of tumor neovasculature by an angiogenesis inhibitor leads to increased apoptosis in tumor tissue.[16,17]

***In vitro* effect of UK1 on the proliferation of endothelial and glioma cells, and tubular structure formation by UK1.** Since UK1 is effective in brain tumor growth, we set up *in vitro* experiments to elucidate the cellular mechanisms of UK1. First, we tested whether purified UK1 is effective in U87 cells as well as endothelial cells. UK1 was assayed for its effect on *in vitro* endothelial cell (HUVEC) and glioma cell (U87, A172 and U373) proliferation stimulated by VEGF (10 ng/mL) employing a [^{3}H] thymidine incorporation assay. As shown in Figure 4a, the growth of HUVECs stimulated by VEGF was greatly inhibited by UK1, while the proliferation of glioma cells including U87 cells was not stimulated by VEGF and their survival was not affected by UK1 at 640 nM and 1280 nM. When the growth of these glioma cells was stimulated by EGF, UK1 did not affect their growth (Fig. 4b). In addition, we determined the effect of UK1 on the tubular structure formation of HUVECs, employing a Matrigel tube formation assay. As demonstrated in Figure 4c, HUVECs, treated with UK1320 nM or 640 nM, formed fewer tubes versus VEGF controls. The suppression level of tubular structure formation was most remarkable at 640 nM. Thereby, these data support the notion that the antitumor activity of UK1 in our glioma model system is mediated by antiangiogenic action.

Discussion

Several angiogenesis inhibitors have been shown to induce a regression of a variety of primary and metastatic tumors in animal models.[17–20] Since it was discovered that kringle molecules, such as angiostatin, have an antiangliogenic activity and antitumor effect, other kringle domains have been studied for their antiangiogenic activity. In comparison with angiostatin kringle domain, UK1 has lower sequence identity (of about 30–40%) of amino acids and does not have binding affinity with lysine residue.[13] We have previously shown that UK1 purified from *E. coli* and pichia, was found to possess antiangiogenic activity.[13,15] Here, we investigated the *in vivo* antitumor effect of UK1, a new antiangiogenic molecule, in a U87 xenograft orthotopic brain tumor model, and its antiangiogenic activity. UK1 suppressed the malignant glioma growth and angiogenesis-related protein expression *in vivo* and inhibited VEGF-stimulated proliferation and tube formation of endothelial cells *in vitro*.

Some kringle domains have been reported to inhibit not only the *in vitro* angiogenesis process but also the *in vivo* glioma tumor growth. For instance, recombinant human angiostatin suppressed the intracerebral 9 L glioma growth[21] and we also reported that recombinant human plasminogen kringle 1–3 (PK1–3) suppressed the U87 glioma growth in nude mice.[16] In this study, we have shown that systemic administration of UK1 (50 mg/kg) suppresses U87 glioma growth by 80%, without any signs and toxicity, showing that UK1 may possibly be as effective as PK1–3 (100 mg/kg, suppressed by 71.2%) even though they were applied under the different conditions of starting treatment and dose. The ATF domain of uPA (aa 4–43) is known as a major binding site for uPAR. As we previously reported, UK1 has more potent antiangiogenic activity than ATF and has a different action mechanism from the ATF domain which blocks the interaction of uPA-uPAR.[14] This result indicates that antiangiogenic activity by UK1 has not proven to be related to the uPA-uPAR system. Recently, stable expression of ATF leads to suppression of *in vivo* brain tumor growth and invasion.[12] uPA receptors exist in both cancer cells and endothelial cells[22] so ATF seems to suppress *in vivo* tumor growth by non-selective cell targeting. Our results showed that UK1 specifically inhibited the proliferation of endothelial cells but not that of glioma cells *in vitro*. Therefore, it could be postulated that *in vivo* antitumor effects of UK1 may be initiated through the inhibition of angiogenesis, and may be followed by suppression of tumor growth and angiogenic factor expression. These indicate that growth and progression of malignant gliomas are closely linked to angiogenesis and UK1 may be a potential molecule for malignant glioma therapy.

Although glioma cells produce several angiogenic factors, VEGF plays the central role in brain tumor angiogenesis.[23] Augmentation of VEGF expression in a growing tumor induces vascular permeability and endothelial cell recruitment into a solid tumor.[24] Inhibition of VEGF-induced activation and its angiogenic cellular function in endothelial cells may be the important reason for the potent *in vivo* antitumor effect by UK1. These findings are further supported by immunohistochemical analysis, displaying significant decrease of vascularity. In addition, the decrease of the pro-angiogenic factor VEGF and angiogenin expression in tumors upon UK1 treatment, also leads to the enhancement of antiangiogenesis in brain tumors. On the other hand, tumor tissue after UK1 treatment increased expression of HIF-1α and caspase 9. These results indicate that UK1-treated mice were more hypoxic than control groups and this eventually

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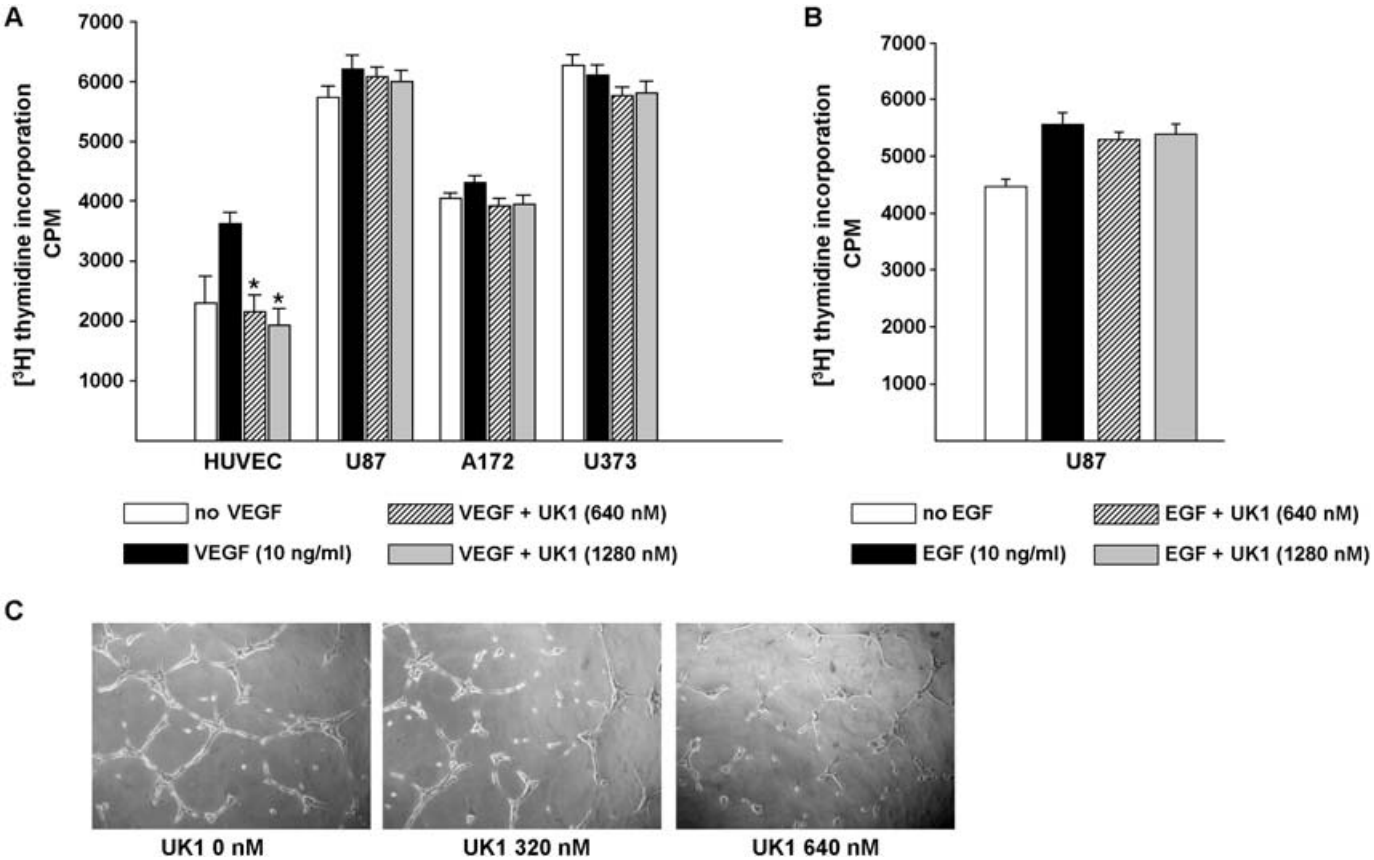

Fig. 4. Anti-proliferative activity of recombinant kringle domain (UK1) on the endothelial cells and glioma cells including U87, A172 and U373 was determined by using a [^{3}H] thymidine incorporation assay induced by vascular endothelial growth factor (VEGF) (A), and Epidermal growth factor (EGF) on U87 (B). (C) Endothelial cell tube formation was inhibited by UK1. Human umbilical vein endothelial cells (4×10^4 cells) were incubated in standard medium, treated with 320 nM UK1, or treated with 640 nM UK1 for 1 h. The data shown are representative of three independent experiments, $^*P < 0.05$ versus VEGF-treated control cells.

induced apoptosis. However, the molecular and cellular inhibition mechanisms of endothelial cells by UK1 are not fully elucidated, so we will try to further investigate the detailed inhibition mechanism by UK1 under VEGF-induced conditions.

In the present study, we started to administer the UK1 at an early stage of tumor development, from day 3 after the i.c. inoculation of U87 tumor cells, at which time the tumor burden was minimal. This may be one of the explanations for the significant *in vivo* antitumor effect of the UK1 treatment in our experiment, which also supports the general concepts of anti-angiogenic therapy preferring the smaller tumor burden or early-stage tumors over advanced or late-stage tumors. A combination of chemotherapy, radiotherapy and other cytotoxic therapies may be an alternative strategy to improving the antitumor effect of UK1.

In summary, UK1 may be a potential antiangiogenic molecule for the treatment of malignant gliomas by suppression of angiogenesis.

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