Targeting Matrix Metalloproteinases and Endothelial Cells with a Fusion Peptide against Tumor

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Abstract

Development of novel therapy for patients with tumor is still a challenge at the present time. We designed a fusion peptide (RK5) with two targets as a novel agent against tumor. The fusion peptide RK5 containing the kringle 5 fragment of human plasminogen and a decapeptide (CTTHWGFTLC) was constructed and expressed in yeast. Matrix metalloproteinase (MMP) activity, proliferation, and migration of endothelial cells were examined *in vitro*, respectively. Angiogenesis, tumor growth, metastasis, and survival time were evaluated in *in vivo* models. Administration of RK5 was delivered by both protein and gene approach. The results showed that RK5 inhibited the activity of MMP-9 and exhibited more inhibitory effects on proliferation and migration of endothelial cells than that of kringle 5 fragment and decapeptide individually. RK5 also inhibited angiogenesis, tumor growth, and metastasis and increased survival time of mice bearing tumor. In addition, the effectiveness of RK5 could be achieved by both protein and gene delivery. In conclusion, RK5 has potential to inhibit tumor growth and metastasis and to prolong survival time of animals bearing tumor. Therefore, fusion peptide RK5 with two targets provides a new design for the development of antitumor drugs and has potential for clinical application. [Cancer Res 2007;67(15):7295–300]

Introduction

Tumor has been viewed as an angiogenesis abnormal disease (1). Angiogenesis is tightly controlled by angiogenic and antiangiogenic systems (2). For the angiogenic system, there are two groups of factors. One stimulates or inhibits endothelial cell proliferation and/or induces endothelial cell elongation, migration, and vascular morphogenesis, and includes vascular endothelial growth factor (VEGF; refs. 3, 4), angiostatin, endostatin, and pigment epithelium-derived factor (5, 6), basic fibroblast growth factor (bFGF; ref. 7), platelet-derived endothelial cell growth factor (8) and the tie and tek receptors (9, 10). The others are involved in the breakdown of basement membranes and extracellular matrix, and include proteases and their receptors such as the members of matrix metalloproteinases (MMP; ref. 11), cathepsins (12), and enzymes involved in the plasmin cascade. It is known that MMPs mediate multiple pathologic processes, including tumor growth and metastasis (13).

For the antiangiogenic system, several native factors have been identified, such as tissue inhibitors of MMPs (TIMP; ref. 14), angiostatin (15), endostatin (16), and thrombospondins (17). Kringle domains of plasminogen also display a strong antiangiogenic activity (18), especially plasminogen kringle 5 (K5; ref. 18). K5 induces apoptosis and cell cycle arrest of proliferating endothelial cells (19). Intravitreal injection of recombinant K5 prevents the development and progression of ischemia-induced retinal neovascularization in a rat model (20), implicating that K5 has a potential therapeutic application in angiogenesis-related diseases.

At present, synthetic protein or peptide treatment shows promising antiangiogenic activity. However, most of the therapeutic protein and peptide exhibit a single target on the angiogenic system, such as either inhibiting proliferation, elongation, migration of endothelial cells, and vascular morphogenesis, or inhibiting enzymes that promote tumor development, growth, and metastasis. The current study investigates the effects of a fusion peptide of decapeptide and K5 on MMP-9 and endothelial cells as an antitumor agent.

Materials and Methods

Construction of yeast and mammalian expression vectors of RK5. The plasmid pET21b-K5 was used as a template to amplify human plasminogen kringle (K5) as described previously (21). K5 cDNA was then subcloned into pPIC9K to form pPIC9K-K5. In addition, K5 cDNA was ligated with cDNA sequence of decapeptide (CTTHWGFTLC) and subcloned into pPIC9K vector at the sites of *Xho*I and *Eco*RI to form pPIC9K-RK5. The pPIC9K-K5 and pPIC9K-RK5 vector was employed and transfected into *Pichia pastoris*. K5 and RK5 were expressed as described previously (21). Decapeptide was synthesized by GL Biochem. RK5 cDNA was also obtained from the pPIC9K-RK5 vector by PCR with the forward primer 5′-GCAGAATTCACCATGGCCCTGTGGATGCGCCT-3′ and reverse primer 5′-GGTCACTCAACATGTGTAGGCTGCGGCTGGGTCAGG-3′. The RK5 cDNA was then subcloned into the mammalian expression vector of pcDNA3.1 at the sites of *Eco*RI and *Hin*dIII to form pcDNA3.1-RK5 for gene therapy.

Administration of mammalian expression vector into animals. Hyaluronidase (359 units/mg; Sigma) was dissolved in sterile saline and injected into the mouse quadriceps at a dose of 36 U per site in a volume of 50 μL. After 1 h, 50 μg of pcDNA3.1-RK5 in 50 μL saline was injected into the same site, immediately followed by square-wave electric pulses using two stainless electrodes placed 5 mm apart at each side of the injected muscle. An electroporator ECM 830 (Genetronics) was used to deliver 16 × 20-ms pulses of 80 V/cm field strength at a frequency of 2 Hz. The treatment was repeated after 1 week. The concentrations of RK5 in the blood serum were measured by RIA using ^{125}I-labeled RK5 on days 3, 8, 13, and 18 after injection.

Cell migration assay. The BD BioCoat Matrigel invasion chamber (BD Bioscience) was used for cell migration assay according to the manufacturer's instructions. Briefly, human umbilical vascular endothelial cells (HUVEC; 2.5×10^4) were seeded onto Matrigel-coated filters in 50 μL serum-free medium with K5, RK5, and decapeptide at a concentration of 40 μg/mL, respectively, and incubated for 0.5 h, followed by adding another 50 μL serum-free medium. In the lower chambers, 10 ng/mL VEGF was added as a chemoattractant. After 24 h incubation, the filters were stained

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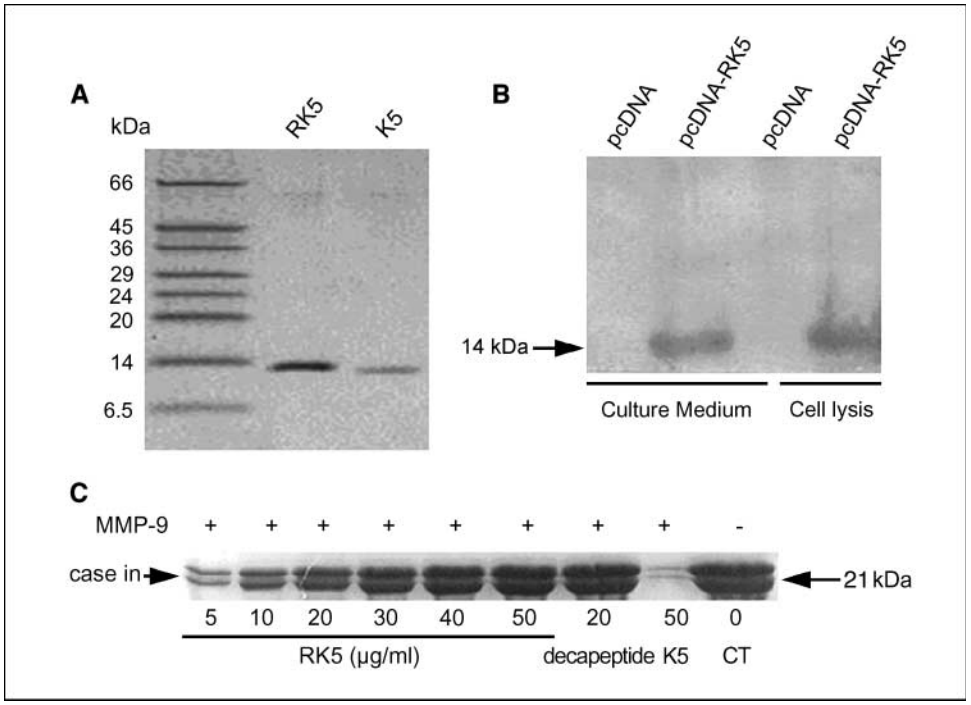

Figure 1. Expression of RK5 in yeast and mammalian cells and its inhibition of MMP-9 activity *in vitro*. After transfection of pPIC9K-K5 and pPIC9K-RK5 vectors into yeast cells, K5 and RK5 were expressed and purified as described in Materials and Methods. *A,* the sizes of K5 and RK5, and the size of RK5 is larger than that of K5. *B,* RK5 peptide extracted from 293T cells after transfection of pcDNA3.1-RK5. The RK5 peptide could be detected in both culture medium and cell lysis by Western blot using the K5 antibody. *C,* effects of purified K5 and purified RK5 and decapeptide on MMP-9 activity. K5 does not inhibit MMP-9 degradation of casein, but decapeptide inhibits MMP-9 activity. RK5 has the similar activity of decapeptide at a concentration of 50 μg/mL, and the dose-dependent inhibition of MMP-9 activity is observed. CT represents no MMP-9 control.

with hematoxylin, and cell migration through the filters was counted under a microscope with magnification of ×400. Migration number was normalized to cell proliferation under the same conditions as control.

Cell proliferation assay. Monolayers of HUVECs were maintained in RPMI 1640 containing 10% fetal bovine serum (FBS) and bFGF (3 ng mL^{-1}). Cell proliferation was evaluated as described previously (20, 21). Briefly, 10^5 cells in 1 mL RPMI 1640 were seeded into 12-well plate coated with fibronectin (1 ng/mL). After 24 h culture at 37°C, the medium was replaced by 0.5 mL of fresh RPMI 1640 containing 5% FBS with different concentrations of K5, RK5, and decapeptide, respectively. Cells were subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after incubation with K5, RK5, and decapeptide for 72 h.

MMP-9 enzymatic assay. About 100 ng of human recombinant MMP-9 (hrMMP-9) from R&D Systems Co. were preincubated with K5, RK5, and decapeptide, respectively, for 60 min at concentrations as indicated in the text. The casein (52 mmol/L) from Sigma Co. was added and incubated for 1 to 4 h at 22°C according to MMP-9 enzymatic assay kit (R&D System Co.). Degradation of casein by MMP-9 was analyzed by electrophoresis on SDS-PAGE.

Mice models of tumor inoculation and peptide treatment. Different mice were employed for different tumor cell inoculation. Male C57BL/6 mice were used for Lewis Lung Cancer (LLC) cells. Mice were 7–8 weeks old and housed in plastic cages with five mice in each cage and were allowed free access to food and water. Animal procedure protocol was approved by the Peking University, which complies with national standards established by the National Council of Animal's Use of China.

LLC cells were suspended in medium with VEGF (5 ng/mL) or bFGF (5 ng/mL) at a concentration of 2×10^6/0.1 mL/mouse (cell viability >85%) for 0.5 h and then injected s.c. into right front axilla of mice. Tumor appearance and growth were evaluated once every 2 days after cell injection. The day of tumor size that reached to 1 cm^3 was designated day 0. K5 (50 mg/kg/day), RK5 (50 mg/kg/day), and decapeptide (25 mg/kg/day) were injected into mice i.p. once a day for 2 weeks. The growth of tumors was measured once every 2 days using vernier calipers. Tumor tissues and lungs from each animal were obtained for histologic staining with H&E and immunohistochemical staining, which were fixed in 4% polyformaldehyde and embedded with paraffin.

Immunohistochemical staining. Tumor tissue sections of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene and

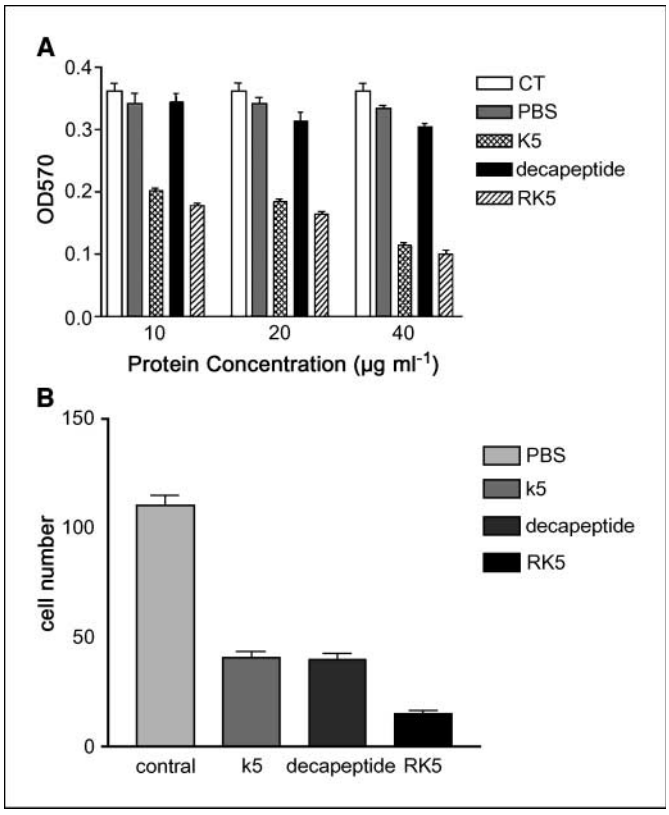

Figure 2. Recombinant fusion peptide RK5-inhibited endothelial cell (HUVEC) proliferation and migration. Purified K5, RK5, and decapeptide were employed to incubate with HUVECs for 24 h. Cell proliferation was determined by MTT assay. *A*, both K5 and RK5 significantly inhibited HUVEC proliferation at concentrations from 10 to 40 μg/mL, but not decapeptide. *B,* effect of purified K5, purified RK5, and decapeptide on HUVEC migration, which was determined by invasion chamber with 10 ng/mL VEGF. All three peptides inhibited HUVEC migration. *Columns,* mean from eight independent experiments; *bars,* SE.

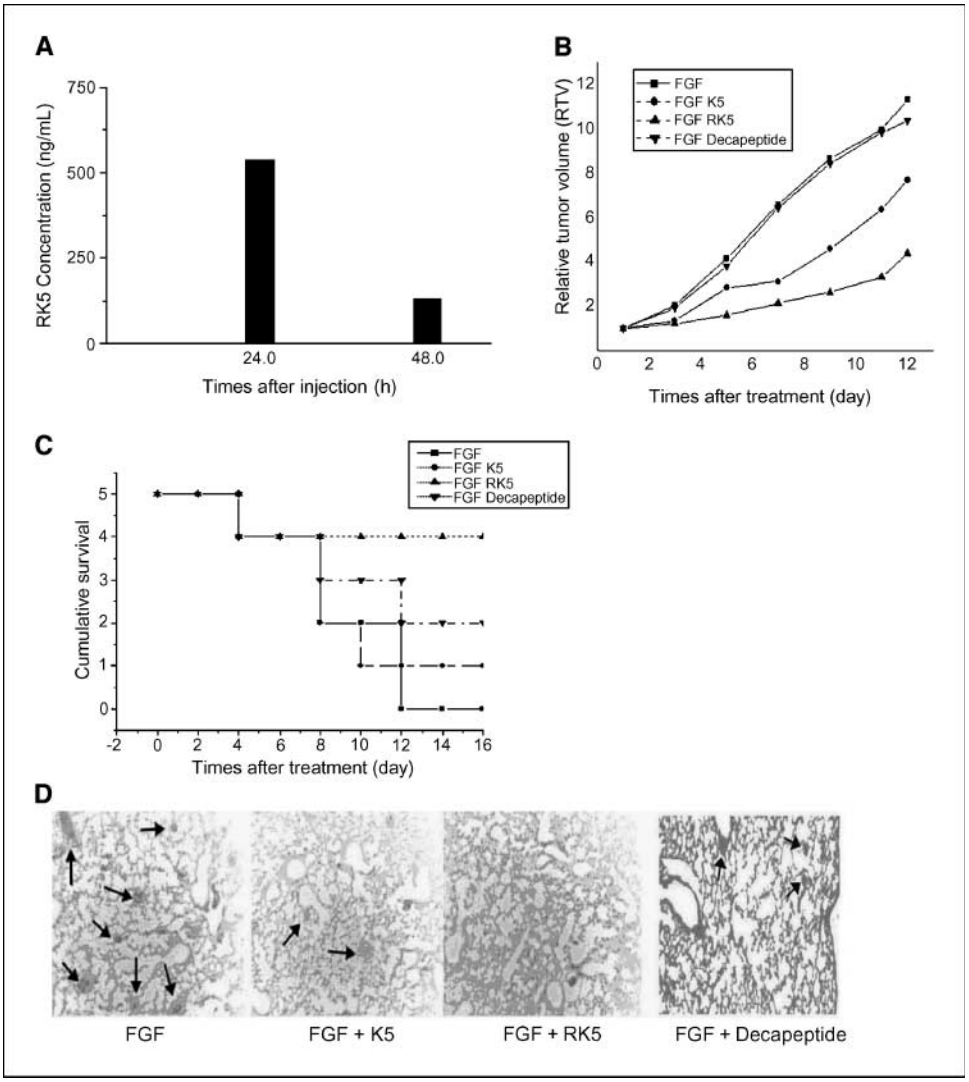

Figure 3. Purified K5 and RK5 inhibited bFGF-induced LLC tumor growth and metastasis and increased survival rate of mice bearing LLC. The serum level of RK5 was first evaluated after i.p. injection of purified RK5. *A*, the amount of RK5 at 24 h is 500 ng/mL and decreased to 100 ng/mL at 48 h. *B,* effects of K5, RK5, and decapeptide on LLC growth in mice. After inoculation of LLC with bFGF (5 ng/mL) into mice and i.p. injection of K5 (50 mg/kg/day), K5 (50 mg/kg/day), and decapeptide (25 mg/kg/day), respectively, both K5 and RK5 inhibited LLC tumor growth, whereas decapeptide did not inhibit tumor growth. RK5-induced tumor growth was greater than K5-induced inhibition. *C,* results of mice survival rate after treatments of K5, RK5, and decapeptide, respectively. Survival rates of mice after treatments were increased, with the highest in mice treated with RK5 and the lowest in mice treated with decapeptide. Lung metastasis of LLC was examined. *D,* K5, RK5, and decapeptide reduced LLC lung metastasis. *Arrow,* metastatic lesions.

rehydrated in graded alcohols. Microvessel staining was carried out with peroxidase-conjugated avidin-biotin method (ultrasensitive S-P Kit from Maixia Bio). Mouse monoclonal antibody against CD34 (Santa Cruz Biotechnology), which recognized the endothelial cell, was incubated with tumor tissue section at a dilution of 1:1,000. After washing, tissue sections were further incubated with secondary biotinylated rabbit antibody against mouse immunoglobulin G (IgG) at a dilution of 1:1,000 (Santa Cruz Biotechnology) and followed by incubation of peroxidase-conjugated avidin at a dilution of 1:1,000. Tissue sections were visualized by incubation with substrates of 3,3′-diaminobenzidine (DAB). Microvessel density in tumor tissue was evaluated by counting the number of vessels in five fields per section at ×200 (0.739 mm^2 per field).

Statistics analysis. Statistical significance of differences was done by employing the ANOVA and Fisher's probable least-squares difference test as

Table 1. The effect of K5, RK5 and Decapeptide on tumor growth (Lewis lung cancer)

Team	Dose (mg/kg/day)	Injection model	Number		Incubate	TV, $x \pm$ SE (mm^3)		RTV, $x \pm$ SE	*T/C* (%)	*P*
			D_0	D_{12}		D_0	D_{12}			
PBS	50	I.p.	5	5	FGF	622.2 ± 303.69	7,014.43 ± 3,743.91	11.27 ± 12.23		0.05
K5	50	I.p.	5	5	FGF	634.816 ± 150.7078	4,866.21 ± 803.27	7.66 ± 5.33	60	0.05
RK5	50	I.p.	5	5	FGF	591.125 ± 194.5934	2,595.04 ± 766.70	4.39 ± 3.94	39	0.05
Decapeptide	25	I.p.	5	5	FGF	598.66 ± 173.6485	6,202.12 ± 1,177.34	10.36 ± 6.78	92	0.05

Abbreviations: TV, tumor volume; RTV, relative tumor volume; *T/C*, tumor/control; D_0, day 0; D_{12}, day 12.

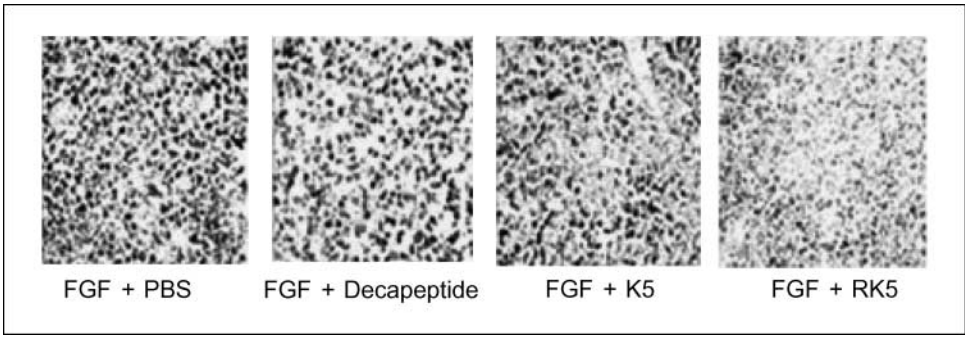

Figure 4. Purified K5 and RK5 inhibited angiogenesis of LLC tumor in mice. LLC inoculation and treatments of K5, RK5, and decapeptide were administrated as indicated in Fig. 3. Tumor tissue sections were fixed and subjected to immunohistochemical staining of CD34 as indicated in Materials and Methods.

post hoc test with Stat View software (version 5.0, SAS Institute Inc.). All measurements are expressed as mean ± SE. Differences were considered to be significant when the P value was <0.05.

Results

Expression of RK5 and its inhibition of MMP-9 *in vitro*. We have constructed a fusion peptide of RK5, which contains the K5 domain of human plasminogen [80 amino acids (aa)] and a 10-aa peptide (decapeptide) that specifically inhibits the activities of MMP-2 and MMP-9 (22). After transfection of RK5 into yeast and 293 cells, the expression and purification of RK5 is shown in Fig. 1. In yeast extract, the purified RK5 protein band was larger than the purified K5 band (Fig. 1*A*). In 293T cells, RK5 could be identified in both culture medium and cell lysis (Fig. 1*B*). The effect of RK5 on the inhibition of MMP-9 was first examined by employing purified RK5 from yeast. As shown in Fig. 1*C*, MMP-9 degradation of casein could be prevented by decapeptide but not by K5. Moreover, RK5 inhibited MMP-9 degradation of casein in a dose-dependent manner.

RK5 inhibition of endothelial cell proliferation and migration. Purified RK5 was employed to incubate with HUVECs for cell proliferation and migration assays. As shown in Fig. 2*A*, with an increase in concentrations of K5 and RK5, there was a decrease in HUVEC proliferation. K5 and RK5 were equally active. However, synthetic decapeptide did not inhibit HUVEC proliferation. HUVEC migration was examined with K5, RK5, and decapeptide. As shown in Fig. 2*B*, K5, RK5, and decapeptide significantly inhibited HUVEC migration. Inhibitory effects of K5 and decapeptide were less than that of RK5.

Inhibition of tumor growth and increased survival of mice bearing LLC by K5 and RK5. To examine the effects of K5 and RK5 on tumor growth and mice survival, we employed the mouse model of LLC. After inoculation of LLC cells pretreated with bFGF into mice, mice were given K5, RK5, and decapeptide, respectively, daily for 8 days. Tumor volume and mice survival were evaluated as shown in Fig. 3. The serum level of RK5 appeared at 24 and 48 h after i.p. injection of RK5 with a peak at 24 h (Fig. 3*A*). Tumor growth was significantly attenuated with the treatment of K5 and RK5 (Fig. 3*B* and Table 1). However, decapeptide could not inhibit tumor growth, whereas RK5 seemed to have a stronger inhibition of tumor growth than K5 did. Moreover, K5, RK5, and decapeptide all improved the survival rate of mice inoculated with LLC cells (Fig. 3*C*), whereas mice treated with RK5 seemed to survive more than mice treated with K5 and decapeptide, respectively. Furthermore, bFGF-treated LLC cells could facilitate the migration of cancer cells to the lung (data not shown). Treatments of K5, RK5, and decapeptide could reduce lung metastasis of LLC (Fig. 3*D*). RK5 showed better inhibition of metastasis than K5 and decapeptide, respectively.

Inhibition of angiogenesis of K5, RK5, and decapeptide. We have shown above that K5 and RK5 could inhibit endothelial cell proliferation, and we further investigated the effects of K5, RK5, and decapeptide on tumor angiogenesis. As shown in Fig. 4 and Table 2, K5 and RK5 dramatically reduced the angiogenesis of the tumor. RK5 treatment seemed to have a stronger inhibition than K5 and decapeptide.

Gene delivery of RK5 inhibits tumor growth and prolongs the survival of mice bearing LLC cells. To examine whether RK5 could be used as gene therapy, we constructed the fusion peptide in a mammalian expression vector with insulin signal peptide that allows RK5 secreting (Fig. 1*A*). Moreover, after the injection of the vector into the right back sural muscle of the mice, the serum level of RK5 was detected at 3 days after injection of RK5, peaked at 8 days, and gradually decreased over 18 days (Fig. 5*A*). Furthermore, the injection of RK5 expression vector into the right back sural muscle of mice inhibited the LLC tumor growth in the mice (Fig. 5*B*) and increased the survival of mice bearing LLC (Fig. 5*C*).

Discussion

K5 of the human plasminogen has widely been used as an antiangiogenesis agent recently (18, 20). It has been shown that K5 induced apoptosis of endothelial and tumor cells through surface-expressed glucose-regulated protein 78 (23). Moreover, K5-engineered glioma cells blocked the migration of tumor-associated macrophages and suppressed tumor vascularization and progression (24). Furthermore, gene transfer of kringle 1-5 caused a significant reduction in vessel density with suppression of tumor growth of the hepatoma cell lines. Kringle 1-5 was also able to prolong the survival period and reduce the number of intrahepatic metastases. Expression of kringle 1-5 protein was detected on hepatoma cells and hepatocytes in the liver. In addition, kringle 1-5 did not alter serum alanine aminotransferase levels and body weights, suggesting the suitability of kringle 1-5 for gene therapy (25). However, combination of K5 with other peptides on tumor vascularization and progression has never been reported. This is the first report of modulation of the activity of the plasminogen protein by a targeting peptide.

Table 2. Microvessel density on tumor

PBS	FGF + K5	FGF + RK5	FGF + Decapeptide
MVD83.78 ± 12.46	50.24 ± 7.88	20.46 ± 8.33	72.83 ± 10.66

Abbreviation: MVD, microvessel density.

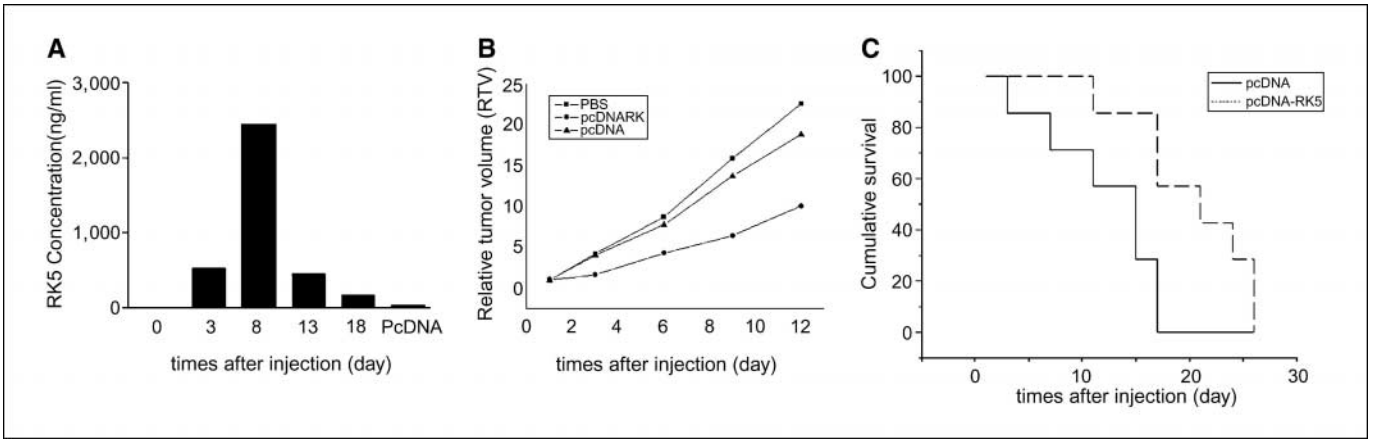

Figure 5. Gene delivery of RK5 inhibited LLC tumor growth and increased survival rate of mice bearing LLC. Inoculation of LLC was the same as indicated in Fig. 3, whereas mammalian expression pcDNA3.1-RK5 was injected into the right back sural muscle of mice as described in Materials and Methods. *A,* serum level of RK5 after injection. The RK5 was detected after 3 d injection and peaked at 8 d. *B,* inhibition of LLC tumor growth. *C,* increase in survival rate of mice after RK5 treatment, respectively.

MMPs are a family of enzymes capable of degrading the constituents of the extracellular matrix and the basement membrane. The two MMPs that closely correlated with the metastatic potential are the MMP-2 and MMP-9, both of which degrade denatured collagens and type IV collagen present in the basement membrane (26). Identification of MMP-9 binding peptide from phage display peptide libraries showed that a decapeptide (CTTHWGFTLC) was a potent inhibitor of MMP-9 (22). Therefore, we designed and prepared a fusion peptide RK5 that contains K5 and decapeptide. The recombinant RK5 showed significant inhibition of endothelial cell proliferation and migration (Fig. 2). Moreover, RK5 was able to inhibit Lewis lung cancer cell growth and metastasis (Fig. 3 and Table 1). Besides, RK5 also inhibits other cancer cell growth and metastasis in the animal model (data not shown), which indicates that the effects of RK5 are not an artifact associated with the use of the LLC cell line only. Our results are the first to report the combination of K5 with the MMP inhibitor for the treatment of cancer, although a combination of VEGF blockade and MMP inhibition for the treatment of experimental human pancreatic cancer has been reported (27). Furthermore, combination therapy with Rho kinase and MMP inhibitor was also able to inhibit angiogenesis and growth of human prostate cancer xenograft (28). Therefore, it should be expected that the recombinant fusion peptide RK5 has a better improvement in the treatment of tumor by targeting both MMPs and endothelial cells. These two targets have been well recognized as a major therapy for cancer. Inhibition of MMPs has been shown to inhibit tumor growth and metastasis (29), whereas attenuation of angiogenesis is well documented to inhibit tumor growth in both tumor models and clinical trials (30). Therefore, the fusion peptide RK5 may exert a synergistic effect on tumor angiogenesis and metastasis.

We also showed that the gene delivery approach could result in the same effect on tumor growth and metastasis as with the protein delivery approach. The inhibition rate for tumor growth by gene transfer was ~55% (Fig. 5), whereas it was ~50–60% by protein delivery (Fig. 3 and Table 1). Compared with the protein delivery approach, gene delivery has some advantages, such as less injection. In our study, only two injections in a week were given during the observation for gene therapy groups, whereas one injection a day for 8 days were given to mice during the observation for protein delivery groups. In addition, gene delivery can keep relatively higher level and longer time of RK5 in the blood as compared with that of protein delivery. Therefore, gene delivery is more clinically applicable than protein delivery. However, clinical application of RK5 gene therapy still needs further investigation.

In conclusion, we have designed and constructed a novel fusion peptide RK5 that can inhibit both MMP activity and endothelial cell proliferation and migration. Moreover, the fusion peptide is able to inhibit tumor growth and metastasis. It represents a new generation of agents against tumor.

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