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NGR Enhanced the Anti-Angiogenic Activity of tum-5

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Tumstatin is an angiogenesis inhibitor. The anti-angiogenic activity of tumstatin is localized to the 54–132 amino acids. NGR motif is a marker of angiogenic endothelial cells. We synthesized the gene fragment encoding the amino acids 45–132 of tumstatin (tum-5) and coupled a NGR (CNGRCVSGCAGRC) motif to the C-terminal of tum-5 (tum-5-NGR). The both were inserted into pQE30 expression vector and expressed in *Escherichia coli*. The anti-angiogenic effects of tum-5-NGR and tum-5 were examined *in vivo*. The results demonstrated the effect of the former was more significant than the latter. After S180 murine cancer xenografts in BALB/c mice were treated with tum-5-NGR or tum-5, tum-5-NGR displayed more significant tumor growth inhibition than tum-5. Binding of tum-5-NGR to normal and tumor tissues was also evaluated. The results showed that the accumulation of tum-5-NGR in tumor tissue was much more than in normal tissues. These data suggest that NGR enhance the anti-angiogenic activity of tum-5.

Key words: anti-angiogenic effect, anticancer drug, NGR motif, tumor growth inhibition, tumstatin.

Abbreviations: EC, endothelial cell; APN, aminopeptidase N; CAM, chick chorioallantoic membrane; SP, streptavidin-peroxidase; IPTG, isopropyl-β-D-thiogalactoside.

Angiogenesis, the development of new capillaries from preexisting blood vessels, facilitates the physiological process of embryonic development, female reproduction, and wound healing (*1*). When angiogenesis occurs, endothelial cells (EC) stimulated by angiogenic factors proliferate and finally form tubular structures at the site to be vascularized (*2*). In a normal adult, there is little angiogenesis except during healing and reproduction. Undesirable angiogenesis plays a critical role in a variety of pathological mechanisms, such as tumor growth, metastasis, diabetic retinopathy and various inflammation diseases (*3–5*). Tumor angiogenesis has become an important area in cancer treatment. Tumor vasculature is a particularly suitable target for cancer therapy because nonmaliganant endothelial cells are genetically stable and therefore unlikely to mutate into drug-resistant variants (*6*).

Tumstatin is an endogenous inhibitor of pathological angiogenesis and suppresses tumor growth (*7–10*). Tumstatin specifically inhibits proliferation of endothelial cells and induces apoptosis *via* an interaction with αVβ3 integrin (*11*, *12*). The full-length tumstatin and fragments containing regions 54–132 and 74–98 amino acids exhibit significant anti-angiogenesis activity. They suppress tumor growth associated human renal carcinoma (786-O) and prostate carcinoma (PC-3) in mouse xenograft models and induced EC-specific apoptosis (*11*, *13*, *14*).

Several peptides that home specially to tumor vascular have been screened *in vivo* (*15*), such as NGR, RGD (Arg-Gly-Asp) and GSL (Gly-Ser-Leu). Coupling anticancer drugs or peptides to the RGD or NGR peptides yield compounds with increased efficacy against tumors and lowered toxicity to normal tissues in mice (*16*, *17*). Aminopeptidase N (APN), also known as CD13, is the receptor for NGR peptides in tumors. NGR peptides can bind an aminopeptidase isoform expressed in tumor vessels, and not other isoforms expressed in normal epithelia or myeloid cells (*18*). Tumor-targeting property of NGR peptides has proven to be very useful for delivering various antitumor compounds to tumors.

To construct a novel anti-angiogensis agent, we synthesized the gene fragment encoding the amino acids 45–132 of tumstatin (tum-5) and coupled a NGR (CNGRCVSGCAGRC) motif to the C-terminal of tum-5 (tum-5-NGR). The tum-5-NGR and tum-5 were expressed in *E. coli*. The both proteins were purified from inclusion bodies. We investigated the anti-angiogenesis effect and the antitumor activity of the two proteins *in vivo*. The results showed that tum-5-NGR have stronger inhibition effects on the formation of vessels and tumor growth than tum-5.

MATERIALS AND METHODS

Animal and Cell Culture—BALB/c mice (4–6 weeks) were fed under specific pathogen-free conditions. S180 cells were cultured at 37°C and in 5% CO_2 atmosphere in DMEM supplemented with 10% heat-inactivated fetal bovine serum containing 100 μg/ml amplicillin and 0.1 mg/ml streptomycin.

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Gene Cloning, Expression and Purification of tum-5-NGR and tum-5—DNA sequencs of the fragmen coding for human tumstatin (amino acid residues: 54–132) and NGR peptide (CNGRCVSGCAGRC) were synthesized. The sequence of whole recombinant gene was designed to include *Sph*I and *Hin*dIII restriction sites. NGR peptide and tum-5 were linked by a soft linker GN. The recombinant gene was subcloned into the sites of *Sph*I and *Hin*dIII of the plasmid pQE30 (Qiagen, USA). The recombinant plasmid was identified using DNA sequencing and named pQE30-tum-5-NGR. Using the same method, the tum-5 DNA was also insert into the pQE30 and named pQE30-tum-5 (Fig. 1). The two plasmids were transfected into DH 5α cells and cultured at 37°C in LB medium supplemented with 100 μg/ml amplicillin. Until the $OD_{600\,nm}$ reached 0.6, 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) were added to induce the expression. The culture was agitation for an additional 4 h, yielding in a final $OD_{600\,nm}$ of 4. Cells were then harvested by centrifugation at $4{,}000 \times g$ for 15 min, washed twice in buffer A (100 mM NaCl, 50 mM Tris-HCl pH 8.0, 1 mM EDTA). The washed cell pellets were lysed in buffer A by sonication and centrifuged. The insoluble fractions were washed with 1% Triton X-100, 2 M urea and the remaining pellet was collected by centrifugation at $15{,}000 \times g$ for 20 min. The isolated inclusion bodies were resuspended in solubilizing buffer B (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl pH 8.0), and incubated at 4°C for 1 h with occasional vortexing. After centrifuging at $15{,}000 \times g$ for 15 min at 4°C, the insoluble debris was discarded. The supernatant was filtered through a 0.45 μm membrane and applied to the nickel (Ni^{2+}) chelate affinity column pre-equilibrated with buffer B (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl pH 8.0) at a flow rate of 0.5 ml/min. Non-specifically bound proteins were removed by extensive wash with buffer B, followed by 25 and 100 mM imidazole washing. Bound proteins were then eluted with 250 mM imidazole in buffer B. The resultant protein was refolded by dialyzing against buffers with decreasing denaturant, firstly against 6 M urea, 0.1 M NaCl, and 20 mM Tris-HCl pH 7.5, secondly against 4 M urea, 0.1 M NaCl, and 20 mM Tris-HCl pH 7.5, then 2 M urea, 0.1 M NaCl, 1 mM EDTA, and 20 mM Tris-HCl pH 7.5, and finally against PBS pH 7.4. Dialysis was performed at 4°C for 16 h with three changes of buffer. After each step, protein samples were centrifuged at $15{,}000 \times g$ at 4°C for 20 min and supernatants were subjected to the next round of dialysis. The refolded protein concentration was determined by the Bradford assay and densitometric analysis. Expression and purification of tum-5 were performed using the similar procedures as described above.

Chick Chorioallantoic Membrane (CAM) Assay—CAM growth was evaluated using a modification of the CAM assay. The air chambers of fertilized 8-day-old chicken embryos were carefully removed. Tum-5-NGR or tum-5, dried on filter paper of 1 × 1 mm^2, were implanted on the CAMs of individual embryos. After a 48-h incubation in 4% CO_2, 96% air at 37°C, CAM tissue was resected and analyzed with a stereomicroscope. The inhibition effects of angiogenesis were determined by the ratio of the number of anti-angiogenic CAMs to the total number of treated CAMs.

Tumor Implantation and Antitumor Studies In Vivo—A total of 5 × 10^5 S180 cells were inoculated in 0.2 ml of serum-free medium right flank subcutaneously of 4–6 weeks old healthy BALB/c mice. When the tumors reached an average volume of about 4 mm^3, the mice bearing too large or too small tumors were eliminated and the left ones were divided into 4 groups [12 animals for each group: PBS, Cyclophosphamide (CTX), tum-5-NGR and tum-5] randomly. CTX (20 mg/kg), tum-5-NGR (10 mg/kg) and tum-5 (10 mg/kg) diluted with 0.9% sodium chloride were injected intravenously once a day. The control group received equal volume of 0.9% sodium chloride each day. Treatment was started on the second day and lasted for a period of 12 days. Tumor size in both groups was measured using a vernier calipers on alternate days, and tumor volume was calculated using a standard formula: tumor volume (mm^3) = width $(mm)^2$ × length (mm) × 0.52. On day 12, animals were sacrificed and tumors excised from each mouse and weighed. The inhibition rates of growth of S180 xenografted tumors were calculated according to the formula: inhibition rate (%) = (1 − tumor weight in test group/tumor weight in control) × 100%.

Binding of tum-5-NGR to Normal and Tumor Tissues—A total of 1 × 10^5 S180 cells were inoculated into BALB/c mice as described above. When tumors reached an average weight of 1.5–3 g, the tumor-bearing mice were divided into 3 study groups (PBS, tum-5-NGR and tum-5) randomly. Tum-5-NGR or tum-5 in 1 mg/kg, or PBS alone as blank control was injected intravenously. After 30 min, the animals were sacrificed and the heart, lung, liver, spleen, kidney and tumor tissues were excised. The tissues were fixed with formalin and embedded into paraffin. The sections (4–5 μm thick) of formalin-fixed (4–6 h), paraffin-embedded tumor tissues, were prepared and coated on polylysine-coated slides. Binding of tum-5-NGR or tum-5 to the tissues were detected by using Streptavidin-Peroxidase (SP) method as follows. Paraffin wax sections were dewaxed in xylene and rehydrated

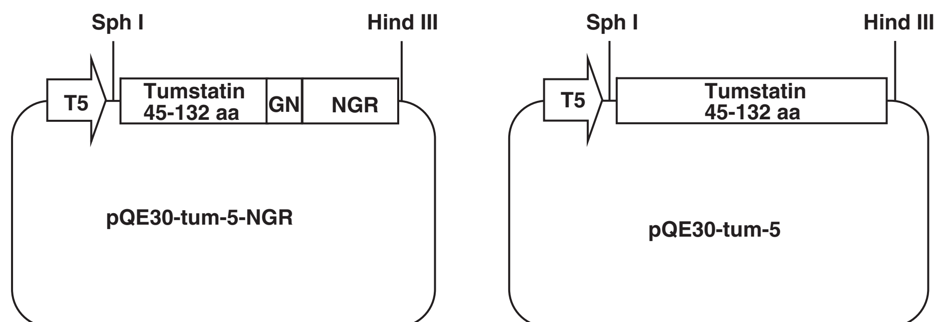

Fig. 1. **Maps of pQE30-tum-5-NGR and pQE30-tum-5 expression vectors.**

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through a series of graded ethanol by standard procedures. Incubating the sections for 30 min in PBS containing 0.3% hydrogen peroxide quenched endogenous peroxidase. After rinsing 3 times (5 min each) with PBS, the sections were incubated with goat serum for 30 min to block non-specific binding. The slides were then allowed to incubated with the mouse anti-His mAb (1:100) overnight at 4°C. After rinsing 3 times with PBS, the sections were then incubated with biotinylated goat antimouse IgG for 10 min at room temperature. The slides were washed again and incubated for 10 min with SP. 3,3′-diamino-benzidine-tetrahydrochloride was dissolved in 2 ml of deionized water containing 0.03% hydrogen peroxide and overlaid on tissue sections for 5–10 min. The slides were washed as described above and counterstained with hematoxylin.

Statistical Analysis—Tumor volumes were expressed as the mean ± SD. Comparison between experimental groups was performed using one-way ANOVA followed by Fisher's protected least-significant difference test. Inhibition rates of CAM and tumor growth were expressed as percentage. Comparisons between experimental groups were tested by chi-square test. Values of $p < 0.05$ were considered significant.

RESULTS

Expression, Purification and Refolding of tum-5-NGR and tum-5—The pQE3-tum-5-NGR and pQE3-tum-5 were transformed into *E. coli* to express the fusion proteins with an N-terminal six-histidine tag. The expressed tum-5-NGR and tum-5 were about 11 kDa and 10 kDa respectively. The expression levels of both were approximately 40% of the total bacteria proteins (Fig. 2A, lanes 2 and 4). But the proteins formed inclusion bodies in *E. coli*. Inclusion bodies were solubilized with 8 M urea and purified by Ni^{2+}-chelating affinity chromatography under denaturing conditions. Then they were refolded by dialysis against gradually decreased concentrations of urea (6 M, 4 M and 2 M) in a buffer containing 0.1 M NaCl. The final yields were 6 mg purified per gram of cell paste (Fig. 2B). The purity of final purified tum-5-NGR shown by Tricine-SDS-PAGE was 95% (data not shown). The purification and refolding of tum-5 were similar to those of tum-5-NGR (Fig. 2B).

Anti-Angiogenic Activity of tum-5-NGR and tum-5—To study the antiangiogenic activity, tum-5-NGR and tum-5 were tested on the CAM (Fig. 3). The proteins, at concentrations of 5–20 μg/embryo, inhibited new embryonic blood vessel growth. However, tum-5-NGR showed a more significant inhibition effect on the newly formed blood vessels than tum-5 ($n = 12$, $p < 0.05$, tum-5-NGR *vs.* tum-5). The inhibition effect was dose-dependent over the range of 5–20 μg/embryo. No obvious inflammation was detected.

Antitumor Effect of tum-5-NGR and tum-5—We compared the antitumor activity of tum-5-NGR with that of tum-5 in S180 tumors (CTX as positive control and PBS as negative control). We found that tum-5-NGR administered daily at 10 mg/kg significantly suppressed the growth of S180 as compared with tum-5 (Fig. 4A). After 12 days of treatment, the weight of tumors treated with tum-5-NGR (10 mg/kg) was significantly lower than that treated with tum-5 (10 mg/kg). The inhibition rate of the both groups was 37.8% and 27% respectively (Fig. 4B). Moreover, all animals in this study appeared healthy with no signs of wasting, and none of the mice died during treatment ($n = 12$, $p < 0.05$, tum-5-NGR *vs.* tum-5).

Binding Study—To investigate whether the enhancing antitumor activity of tum-5-NGR is related to NGR, binding of tum-5-NGR to normal and tumor tissues was detected at 30 min post-injection by immunohistochemistry. Normal tissues sections including heart, lung, liver, spleen and kidney from tum-5–injected mice were detected stronger immunoreactivity positive signals than those from tum-5-NGR. On the contrary, tumor tissues sections from tum-5-NGR–injected mice were detected stronger immunoreactivity positive signals than those from tum-5 (Fig. 5). No immunoreactivity positive signals were detected in the sections from PBS-injected mice.

DISCUSSION

As potential anti-tumor drug candidates, tumstatin and its derivations having been researched intensively. They have been expressed and purified in *E. coli* and human 293

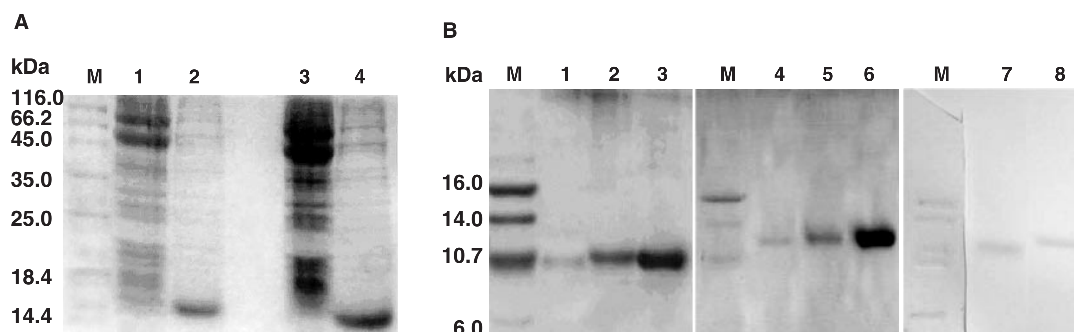

Fig. 2. **Expression, purification and identification of tum-5-NGR and tum-5.** (A) Twelve percent of Tricine-SDS-PAGE analysis of the expression of tum-5-NGR and tum-5. M, Protein molecular weight marker; lane 1 and 3, whole cell lysate of pQE30-tum-5-NGR and pQE30-tum-5 before induction; lane 2, whole cell lysate of pQE30-tum-5-NGR after induced; lane 4, total lysate of DH5α pQE30-tum-5 after induced. (B) Purification and identification of tum-5-NGR and tum-5. Proteins were identified by Western blot with anti–His-tag antibody. M, protein molecular weight markers; Lanes 1–3, tum-5-NGR eluted with 25 mM, 100 mM and 250 mM imidazole respectively; lanes 4–6, tum-5 eluted with 25 mM, 100 mM and 250 mM imidazole respectively. Lane 7, tum-5-NGR, corresponding to lane 3, was detected by Western Blot; Lane 8, tum-5, corresponding to lane 6, was detected by Western blot.

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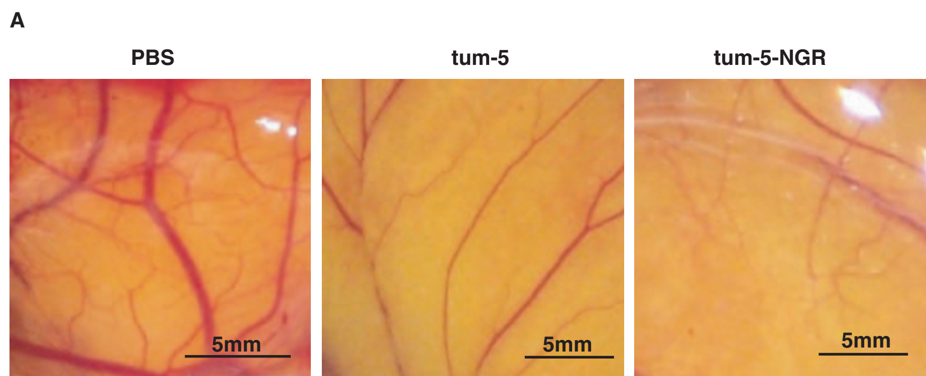

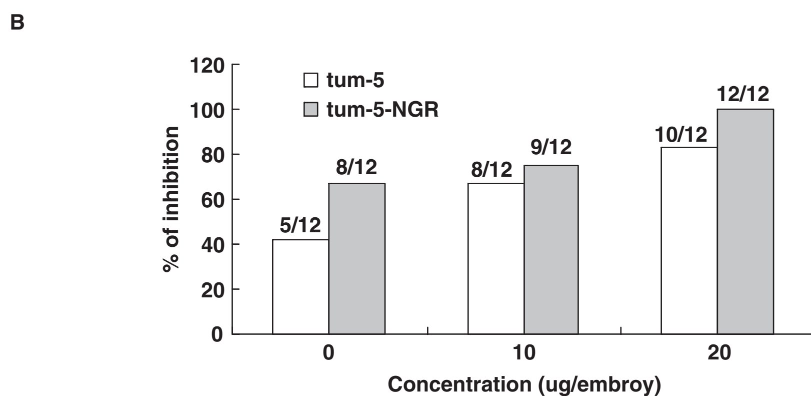

Fig. 3. **Inhibition of angiogenesis by tum-5-NGR.** (A) Filter paper containing various amounts of tum-5-NGR or tum-5 was implanted on CAMs of 8-day chicken embryos. After 48 h, CAM tissue was resected, analyzed and photographed under a stereomicroscope. Bar = 5 mm. (B) The numbers of anti-angiogenesis CAMs over the total number of CAMs tested at various concentrations of tum-5-NGR or tum-5 are indicated above each bar.

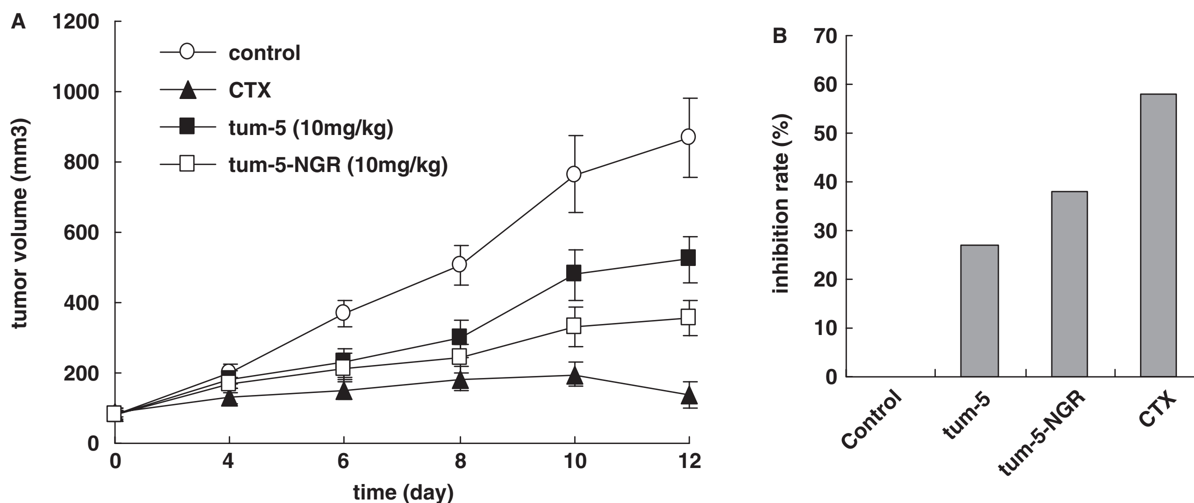

Fig. 4. **Antitumor effect of tum-5-NGR and tum-5 on murine tumor model.** (A) Mice were treated with drugs daily after tumor implantation and dose indicated in each panel (12 animals per group). The tumor volumes were measured every two days. (B) At the end of this study, all of the tumor-bearing animals were sacrificed, tumor weight was determined and the inhibition rates were calculated.

embryonic kidney cells respectively (*19–22*). In this study, a new recombinant fusion protein tum-5-NGR was constructed and expressed in *E. coli*. The protein is composed of tum-5 and NGR peptide. We coupled the cyclic peptide NGR motif to the C terminus of tum-5 for two reasons: (i) The N-terminal 9 amino acids of tum-5 can increase the expression efficiency in prokaryotic expression system (*21*), (ii) NGR peptide fused to C terminus of tum-5 may be of advantage to the exposure of NGR motif on the molecular surface.

Like the full-length tumstatin, the fragments containing regions 54–132 and 74–98 amino acids had significant anti-angiogenesis activity (*23*). APN/CD13, the receptor of NGR peptide, has proved to play a critical role in the formation of angiogenesis (*24*). We intend to increase the anti-angiogenic activity of tum-5 by coupling a NGR peptide to the protein. The CAM assay showed that tum-5-NGR had stronger suppression effect on embryonic vascular formation than tum-5. This suggests that NGR peptide enhance the anti-angiogenesis of tum-5. We speculated that NGR

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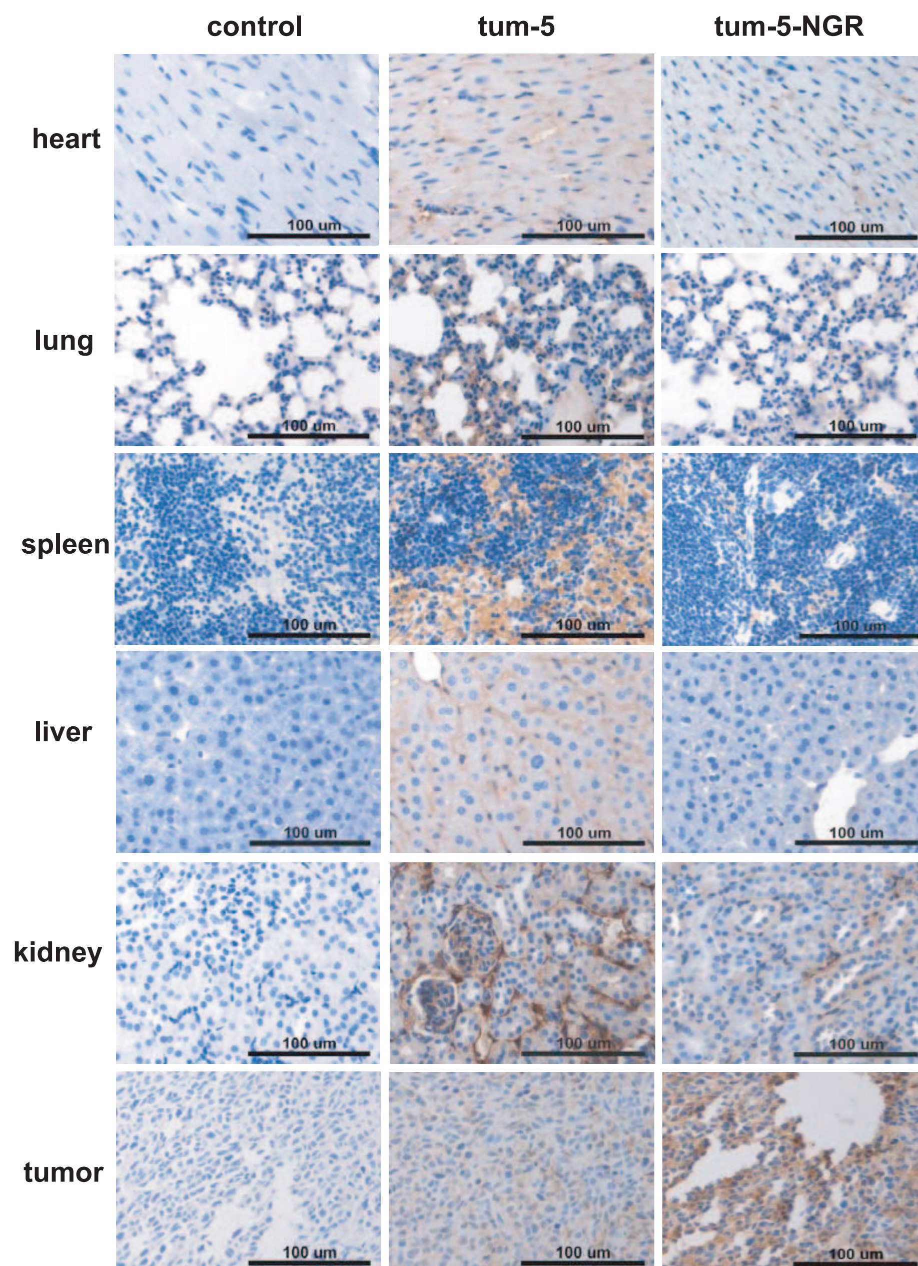

Fig. 5. **Binding of tum-5-NGR to normal and tumor tissues.** The dissected normal and tumor tissues, 30 min after injection, were immunostained with anti-His antibody. The representative photographs were taken under light microscope. Original magnification, ×200. Bar = 100 μm.

binding to APN might block the function of APN, which in turn contributed to the anti-angiogenesis of tum-5.

Tumstatin demonstrates genetic evidence for a physiological role in the negative regulation of tumor growth progression in mice (*25*, *26*), such as human renal carcinoma and prostate carcinomas in mouse xenograft models. Since tum-5-NGR showed stronger anti-angiogenic activity than tum-5, we evaluated the anti-tumor activity of tum-5-NGR in mice bearing tumors. The results showed that the inhibition effect of tum-5-NGR at 10 mg/kg/day on tumor growth was markedly higher than that of tum-5 at the same dose. This suggests that NGR increase the therapeutic index of tum-5. To further insight into the tumor-targeted capacity of NGR motif, the accumulation of tum-5-NGR in normal and tumor tissue at 30 min after injection were observed by immunohistochemistry. It indicated that the accumulation of tum-5-NGR in tumor tissue was much more than in normal tissues. In addition, the accumulation of tum-5-NGR in tumor tissue was more than tum-5, whereas the accumulation of tum-5-NGR in normal tissues was less than that of tum-5. The result strongly suggests that NGR motif has tumor-homing effect.

The current study indicated that coupling NGR peptide to tum-5 increase the selective binding of tum-5 to tumor tissue and the local efficacy of tum-5 against tumor. It suggests that tum-5-NGR may be an effective angiogenesis inhibitor and a potential candidate for the treatment of cancer.

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