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Suppression of tumor growth by novel peptides homing to tumor-derived new blood vessels

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Abstract Novel peptides homing to angiogenic vessels were recently isolated from a phage-displayed random pentadecapeptide library. One of the isolated peptides, ASSSYPLIHWRPWAR, significantly suppressed the migration of VEGF-stimulated human umbilical vein endothelial cells. Dendoric ASSSYPLIHWRPWAR-peptide suppressed the formation of new blood vessels in dorsal air sac model mice. Furthermore, ASSSYPLIHWRPWAR-peptide and the fragment peptides containing WRP, which is revealed to be an epitope sequence, significantly suppressed the tumor growth, although 15-mer shuffled peptide derived from ASSSYPLIHWRPWAR and pentapeptides with alanine substitution of each residue of WRP did not. Taken together, ASSSYPLIHWRPWAR-peptide may cause tumor dormancy through inhibition of angiogenesis, and the WRP sequence may be the minimal and essential sequence for this activity.

Key words: Phage-displayed peptide library; Angiogenesis; Drug delivery system; WRP; Tumor dormancy

1. Introduction

Antiangiogenic agents induce tumor dormancy, suppress metastases, and essentially cause little side effects [1,2]. Therefore, many antiangiogenic agents have been developed one after another, and preclinical and clinical studies are now in progress. The angiogenic vasculature would not be expected to acquire drug-resistance. In fact, antiangiogenic-scheduled chemotherapy combined with an angiogenesis inhibitor eradicated even the drug-resistant tumor cells [3]. On the other hand, vascular targeting has become a focus of interest, since certain drugs or drug carriers first meet the tissue vasculature and angiogenic vasculature has properties different from those of the preexisting systemic vasculature [4,5]. Specific ligands against molecules on the angiogenic endothelia are considered to be useful for active targeting to tumor angiogenic vasculature.

Recently, we prepared peptides specific for tumor angiogenic vasculature by using a phage-displayed peptide library for delivering antiangiogenic agents or anticancer agents more effective to the angiogenic site, since such libraries are useful for obtaining specific peptides bound to appropriate target molecules [6–8]. In brief, we injected a phage-displayed peptide library into angiogenesis model mice. Phage clones that accumulated in angiogenic blood vessels formed by the dorsal air sac (DAS) method [9] were isolated. The advantage of this method is that the selected phages have the ability to bind only to angiogenic vessels, not to tumor cells. The peptide sequences of the phage clones thus obtained were different from any reported sequences obtained by in vivo biopanning with tumor-bearing mice [10]. The obtained peptides, ASSSYPLIHWRPWAR, DRWRPALPVVLFPLH, and PRPGAPLAGSWPGTS, presented on selected phages actually accumulated in the tumor tissues of two different tumor cell types [10]. In the present study, we examined the effect of these isolated peptides on angiogenesis and observed that one of these peptides, ASSSYPLIHWRPWAR, suppressed angiogenesis possibly through inhibition of endothelial cells migration. Furthermore, the synthetic peptides based on the isolated phages had the ability to suppress angiogenesis and tumor growth.

2. Materials and methods

2.1. Tumor implantation

Highly metastatic murine B16BL6 melanoma cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 medium containing 10% fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS, USA). After harvesting of the cells, 1.0×10^6 cells were carefully injected subcutaneously into the posterior flank of 5-week-old C57BL/6 male mice (Japan SLC Inc.). Meth A sarcoma cells were grown in 5-week-old Balb/c male mice (Japan SLC Inc.) under an appropriate schedule, and were similarly injected (1.0×10^6 cells/mouse) into the posterior flank of Balb/c mice. The animals were cared for according to the animal facility guideline of the University of Shizuoka.

2.2. Inhibitory effect of synthetic peptide against phage accumulation

Peptides were synthesized by use of Rink amide resin (0.4–0.7 mmol/g) and a peptide synthesizer ACT357 (Advanced ChemTech), resulting in an amide at the carboxyl terminus. Preparation of M13 phages expressing unique amino acid sequence and in vivo phage accumulation assay were done as described previously [10]. Purified phage clone (5×10^8 cfu) and 0.25 μmol of each synthetic peptide was coinjected into tumor-bearing mice when the tumor size became about 10 mm in diameter. Four minutes after injection, the tumor tissue was dissected, minced, and homogenized. The phages in homogenate were recovered by infecting *Escherichia coli* K91KAN and titrated.

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2.3. Inhibitory effect of synthetic peptide against migration of human umbilical vein endothelial cells (HUVECs)

HUVECs (BioWhittaker, Walkersville, MD, USA) cultured in endothelial cell basal medium (EBM, BioWhittaker) were labeled by an incubation with 3′-*O*-acetyl-2′,7′-bis(carboxyethyl)-4 or 5-carboxyfluorescein, diacetoxy-methylester (BCECF-AM; Dojindo Laboratories, Kumamoto, Japan) for determining the migration capacity by a method as previously described [11]. After washing, 5×10^4 labeled cells in EBM were introduced into a culture insert having a fluorescence blocking micropore membrane (FBM, Falcon HTS Fluoro-Blok™ Inserts, Becton Dickinson) precoated with matrigel (Becton Dickinson). Each culture insert was set into a well of a 24-well plate containing 10% FBS–EBM. Then, an appropriate amount of rhVEGF (Becton Dickinson) and synthetic peptides were added to the upper side of the insert. After a 16-h incubation, the FBM was examined under a fluorescence microscope for counting the number of cells invaded.

2.4. Inhibitory effect of dendoric synthetic peptide on in vivo angiogenesis

Meth A sarcoma cells (1.0×10^7 cells/ring) were transferred into a chamber ring of which both sides were covered with Millipore filters having a 0.45-μm pore size (Millipore Co., Bedford, MA, USA). The chamber rings were dorsally implanted into 5-week-old Balb/c male mice. Dendoric synthetic 15-mer peptides (30 mg/kg/day) were injected intravenously into a tail vein of the ring-bearing mice every day starting at 1 day after ring implantation. Four days after the implantation, the mice were sacrificed, and angiogenic vessels formed at the dorsal skin were photographed.

2.5. Therapeutic experiment and statistical analysis

Synthetic 15-mer peptides and fragment peptides (20 mg/kg/day) were injected subcutaneously into a site neighboring the tumor of mice every day starting at 1 day after tumor implantation. Injection sites of peptides were carefully selected to be at least 5 mm distant from the tumor. The weight of each mouse, an indicator of side effect, and size of the tumor were monitored every day after initiation of peptide administration. Tumor volume was calculated as described previously [12]. Variance in a group was evaluated by the *F*-test, and differences in mean tumor volume were evaluated by Student's *t*-test.

3. Results and discussion

Two distinct phage clones isolated with a homing capacity to tumor new blood vessels presented ASSSYPLIHWRPWAR and DRWRPALPVVLFPLH sequences. Since both sequences have a common tripeptide sequence, WRP, this sequence may serve as an epitope responsible for the homing to the angiogenic site. To confirm the capacity of the synthetic peptides to accumulate in tumors and to examine the importance of WRP, we coinjected selected phage clones and corresponding synthetic peptides into B16BL6 melanoma-bearing mice. As a result, all tested 8- and 5-mer fragment peptides containing WRP sequence inhibited the accumulation of phages expressing original 15-mer peptides (Fig. 1). These inhibitory effects indicate that these peptides may interact with a specific molecule(s) through a common sequence, namely, WRP.

Next, we examined whether the angiogenic-specific peptides isolated from the phage-displayed library would have some biological effects on angiogenesis-related events or not. Since solubility of ASSSYPLIHWRPWAR is superior to DRWRPALPVVLFPLH, we used ASSSYPLIHWRPWAR in the following study. At first, we examined the effect of the peptide on the invasion activity of endothelial cells that had been activated by VEGF, since VEGF is known to activate motility and proliferation of endothelial cells. In fact, VEGF activated invasion and proliferation of HUVECs in a dose-dependent manner (data not shown). As shown in Fig. 2A, the ASSSYPLIHWRPWAR-peptide inhibited invasion of VEGF-stimulated HUVECs in a dose-dependent manner, and HUVEC migration declined to the background level in the presence of 1 μmol ASSSYPLIHWRPWAR-peptide. In contrast, treatment with the shuffled peptide of ASSSYPLIHWRPWAR, i.e. SAYPALSWSHRRIWP, which sequence had been determined by the randomization of the parental peptide sequence, did not show any suppression of HUVEC migration. The suppression of HUVEC invasion by the treatment with ASSSYPLIHWRPWAR-peptide might not be due to peptide-induced damage of HUVEC, since no cytotoxic action of the ASSSYPLIHWRPWAR-peptide against HUVECs was observed at least up to a 100-μmol concentration of the peptide (data not shown).

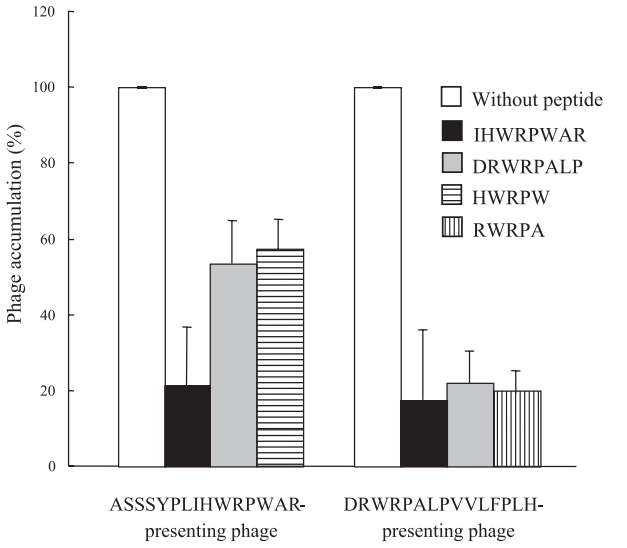

Fig. 1. Inhibitory effect of WRP-containing fragment peptides against affinity-selected phage accumulation in tumor tissue. Phage (5×10^8 cfu) and fragment peptides (0.25 μmol) were intravenously coinjected into tumor-bearing mice ($n=3$). 4 min after the injection, the deeply anesthetized mice were snap-frozen in liquid nitrogen, and their tumor tissue was then dissected. The accumulated phages at the tissue were recovered and titrated. Amino acid sequences of injected peptides are indicated.

We next examined the inhibitory effect of the peptides on angiogenesis in vivo. Dendoric ASSSYPLIHWRPWAR, $(ASSSYPLIHWRPWAR)_8K_4K_2K$, but not dendoric shuffled peptide, showed suppression of tumor angiogenesis in the DAS model (Fig. 2B).

Since the peptides isolated from the phage library showed specific affinity for tumor angiogenic sites, inhibited invasion of HUVECs, and inhibited angiogenesis, these peptides are expected to cause tumor dormancy via the suppression of the angiogenic process. Therefore, we examined the effect of the peptides on tumor growth in vivo. Pentadecapeptides and fragment peptides (20 mg/kg/day) were injected intravenously into mice bearing Meth A sarcoma, and the tumor growth inhibition by these peptides was investigated. As shown in Fig. 3A, synthetic ASSSYPLIHWRPWAR peptide, but not shuffled peptide SAYPALSWSHRRIWP, caused potent suppression of tumor growth: tumor growth was inhibited 91.1% determined at day 11, the final day of the treatment (significantly different from the control or shuffled peptide-treated, $P<0.01$). Similar results were obtained by the use of C-ter-

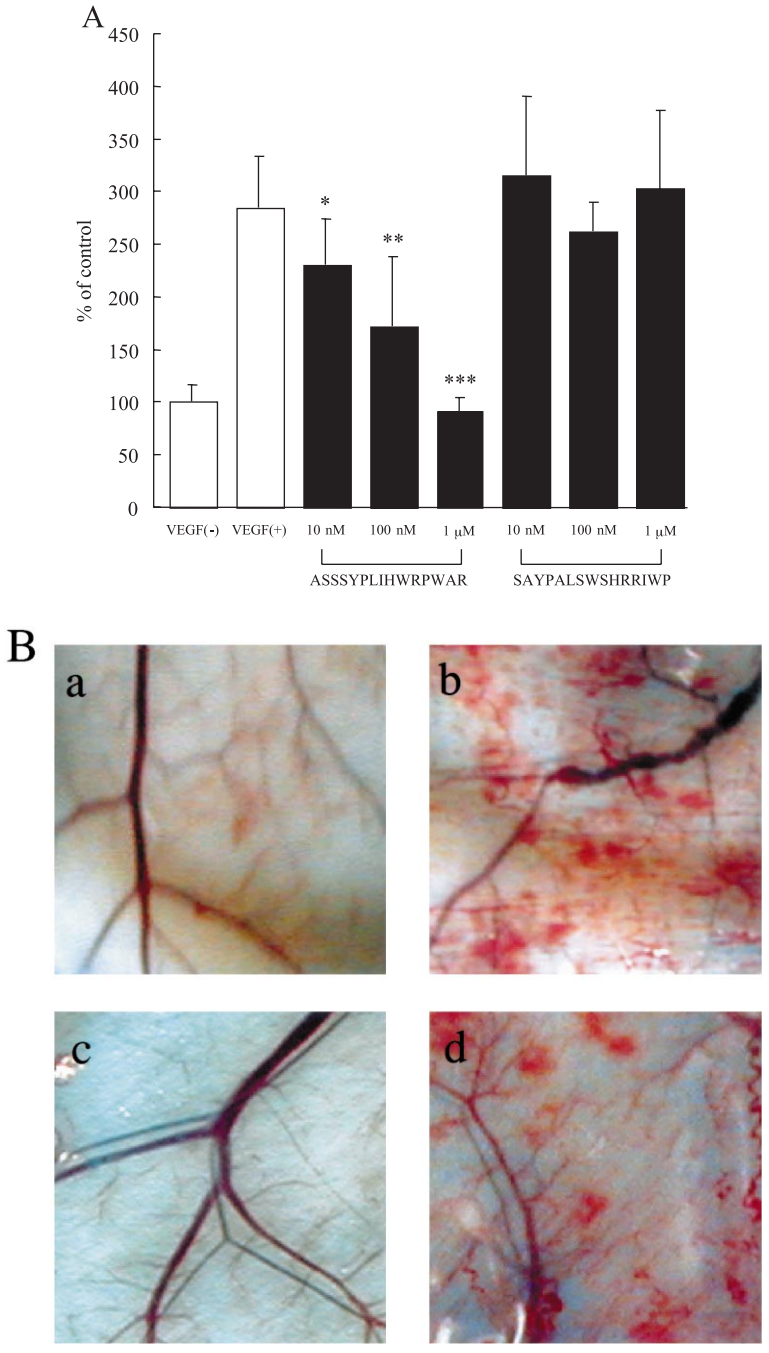

Fig. 2. Inhibition of HUVEC migration and in vivo angiogenesis by ASSSYPLIHWRPWAR peptide. A: BCECF-AM-labeled HUVECs were added to matrigel-coated FluoroBlok inserts at a density of 50 000 cells/insert. The upper chamber was supplemented with 10 ng/ml rhVEGF or without it. ASSSYPLIHWRPWAR peptide and shuffled peptide were added to the upper chamber at a concentration of 10 nM, 100 nM, or 1 μmol, respectively, in the presence of rhVEGF. The migration was determined as described in Section 2. Significant differences from rhVEGF treated group without peptide are indicated (*$P<0.05$, **$P<0.01$, ***$P<0.001$). B: Chamber rings containing Meth A sarcoma cells (1.0×10^7 cells/ring, a, b, d) or medium alone (c) were dorsally implanted into 5-week-old Balb/c male mice. Dendoric synthetic 15-mer peptide, ASSSYPLIHWRPWAR (a), dendoric shuffled peptide, SAYPALSWSHRRIWP (b), (30 mg/kg/day) or saline (c,d) was injected intravenously via a tail vein of the ring-bearing mice every day starting at 1 day after the ring implantation. Four days after the implantation, the mice were sacrificed, and angiogenic vessels formed in the dorsal skin were then examined.

minus octapeptide, IHWRPWAR (82.1% reduction, significantly different from the control or shuffled peptide-treated, $P<0.01$), and HWRPW pentapeptide (88.6% reduction, significantly different from the control or shuffled peptide-treated, $P<0.01$). N-terminus 8-mer ASSSYPLI, however, did not show any suppression of tumor growth. Thus WRP-containing sequences, at least HWRPW, may be important for tumor growth suppression. After the last day of administration, tumor regrowth was observed (see after day 12 of Fig. 3A), suggesting that suppression of angiogenesis may be cancelled. In other words, ASSSYPLIHWRPWAR and the fragment peptides containing the WRP sequence might cause tumor dormancy during treatment. The DRW-RPALPVVLFPLH peptide also showed suppression of tumor

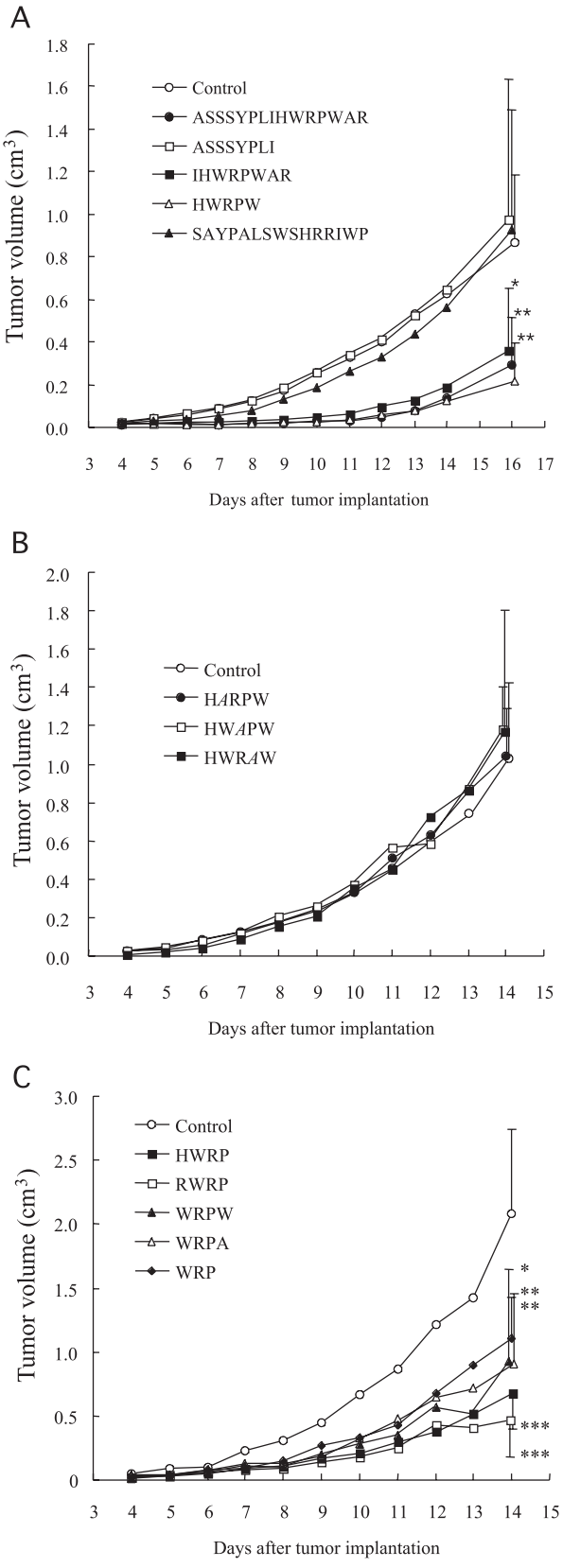

Fig. 3. Suppression of tumor growth in Meth A sarcoma-bearing mice by treatment with synthetic peptides. 5-week-old Balb/c male mice ($n = 6$) were implanted subcutaneously with Meth A sarcoma into their left posterior flank. At days 1–10 after tumor implantation, they were injected subcutaneously with various fragment peptides (A,C) or alanine-substituted peptides (B) at a dose of 20 mg/kg. Tumor volume was determined as described in Section 2. Standard deviation bars are shown only for the last points for the sake of graphic clarity. Significant differences from control are indicated ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

growth, but the activity of the peptides was slightly poorer than that of ASSSYPLIHWRPWAR (data not shown).

To confirm the importance of the WRP sequence, we examined the tumor growth-suppressing activity by using alanine-replaced pentapeptides, namely, HARPW, HWAPW, and HWRAW. None of the three alanine-replaced peptides of HWRPW suppressed the tumor growth (Fig. 3B), indicating that WRP is an essential sequence for the tumor growth suppression. In fact, the WRP tripeptide also inhibited tumor growth, although the effect was less potent than that of HWRPW (Fig. 3A,C). These results suggest that WRP is the minimum and essential sequence for inducing tumor dormancy, although the flanking sequence of WRP may also important for modulating the activity. None of the peptides tested showed any cytotoxic action in vitro against Meth A sarcoma cells, nor caused body weight changes of animals in vivo as an indicator of the side effects (data not shown). In these experiments, we started treatment of tumor-bearing mice 1 day after tumor implantation. We also examined the timing of initiation of treatment by the use of ASSSYPLIHWRPWAR and fragment peptides (20 mg/kg/day), and observed little suppression of tumor growth when the tumor sizes were more than 4 mm in diameter at the initiation of treatment (data not shown). These data may also support the idea that the peptides affect angiogenesis rather than the tumor cells directly.

Angiogenesis is a critical event for tumor growth and metastasis, and angiogenesis-suppressing agents may thus provide a new modality of tumor treatment. In fact, angiostatin, endostatin [13], and the cleaved conformation of the serpin antithrombin [14], which were discovered as endogenous angiogenesis inhibitors, suppressed tumor growth. In the present study, we identified a novel peptide having antiangiogenic activity. The sequence of this peptide has not been isolated in other tumor screenings. A homology search of ASSSYPLIHWRPWAR revealed that the HWRPW sequence in the ASSSYPLIHWRPWAR peptide was identical to a part of the sequence of Flt-4, which is a receptor for VEGFs. VEGF-C, a subclass of VEGF that is reported to be an angiogenesis stimulator [15], is known to bind to Flt-4 as well as to Flk-1/KDR, another VEGF receptor. However, the importance of the HWRPW sequence for the interaction of VEGF-C with its receptors is not clear at present. Although the target molecule(s) of the WRP-containing peptides is unclear at present, angiogenic vasculature-specific peptides, namely, WRP-related peptides, shown here may be useful as a lead compound for the development of new antiangiogenic agents. Moreover, the peptides may also provide tools for active targeting of tumor tissues: direct conjugation of the peptides with anticancer agents or angiogenesis inhibitors, or modification of drug carriers with the peptides, may increase the local concentration of agents in tumor angiogenic sites after administration.

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