Targeting of Tumor Blood Vessels: A Phage-Displayed Tumor-Homing Peptide Specifically Binds to Matrix Metalloproteinase-2-Processed Collagen IV and Blocks Angiogenesis In vivo

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Abstract
Proteolytic degradation of the basement membrane by the matrix metalloproteinase-2 and -9 is an essential step in tumor angiogenesis. On proteolytic degradation, cryptic sites in collagen IV are formed, which serve as a migration signal for endothelial cells and are specific for angiogenic blood vessels. The aim of this study was to generate peptides that bind specifically to proteolytically processed collagen IV and to test whether these peptides accumulate in tumor vasculature and are able to block angiogenesis. To obtain such peptides, we performed a combined in vivo and in vitro phage display screen using a recombinant phage-displayed peptide library. We found a phage displaying the peptide sequence TLTYTWS that specifically binds to collagen IV modified by matrix metalloproteinase-2. We then tested the ability of the phage to bind to the vasculature in xenograft tumors and found indeed a significant accumulation of the phage in tumors but not in control organs. The tumor homing of the TLTYTWS phage is specific, as it can be blocked by coinjection chemically synthesized cognate peptide. Moreover, TLTYTWS peptide inhibits angiogenesis in an in vivo assay in a concentration-dependent manner and significantly reduces endothelial differentiation in vitro. In conclusion, we report about a novel tumor-homing peptide that specifically binds to proteolytically processed collagen IV, accumulates in tumors, and blocks angiogenesis. This peptide may be a new useful tool for diagnostic and therapeutic procedures in oncology. (Mol Cancer Res 2009;7(7):1078–85)

Introduction
The vascular beds of individual tissues differ in structure and metabolic function as well as in the expression of organ-specific adhesion molecules. In particular, molecules are expressed in the vasculature of tumors that are not present in normal blood vessels and therefore may serve as targets for novel tumor therapies (1, 2). It has been shown that peptide libraries displayed on phage can be screened in vivo and in vitro for phage homing to a specific target. Several peptides capable of homing to tumor vasculature were isolated in this manner (3, 4). Moreover, the therapeutic efficacy of these tumor-homing peptides coupled to cytotoxic drugs has been shown in preclinical models of cancer (5). Therefore, the pathophysiology of tumor vasculature is a promising study area for the discovery of novel target molecules for specific tumor therapies. Within the tumor vasculature, not only endothelial cells but also molecules within the extracellular matrix may constitute interesting target structures.

It is generally accepted that cleavage of the basement membrane of preexisting blood vessels by matrix metalloproteinases (MMP) and other proteases is essential for tumor angiogenesis (6–9). The basement membrane is mainly composed of the collagen IV network, the laminin network, nidogen, heparan sulfate proteoglycans, and perlecain molecules (10). Due to their differential expression and localization in different tissues, collagen IV and laminin isoforms form tissue-specific basement membranes (10, 11). In blood vessels primarily, the isoform [α1(IV)]2 [α2(IV)]1 of collagen IV is found (10, 12). The basement membrane of blood vessels not only serves as a solid support for endothelial cell adhesion but also plays an active role in the regulation of angiogenesis. For example, heparan sulfate proteoglycans and laminins present in the basement membrane are capable of binding growth factors such as vascular endothelial growth factor and fibroblast growth factor (FGF) and thereby block proangiogenic effects of these mediators (13, 14). It also contains molecules that interfere with endothelial cell adhesion, such as thrombospondin, negatively regulating endothelial cell migration. Some basement membrane proteins also avidly bind to cell adhesion molecules (10) and thereby induce quiescence of endothelial cells in blood vessels (13, 15). The MMP family consists of 23 different zinc-dependent endopeptidases, which cleave collagen, laminin, proteoglycans, as well as several other proteins (16). Two of these MMPs (MMP-2 and MMP-9) cleave gelatin and therefore are termed gelatinases. They also cleave collagen IV, laminin, and other noncollagenous extracellular matrix proteins (16–18). Whereas MMP-9 is not active on intact collagen, it is a subject of debate under which conditions MMP-2 is capable of cleaving intact collagen IV (19, 20). Nevertheless, the importance of MMP-2 and MMP-9 in angiogenesis is widely accepted (21). Proteolytic degradation of the basement membrane by MMPs promotes angiogenesis, as sequestered growth factors are
liberated and bioactive fragments of basement membrane components are generated (22, 23). One of these bioactive fragments is proteolytically modified collagen IV itself: proteolytic degradation leads to the exposure of cryptic sites, which are normally concealed by the triple helical structure of collagen IV. These cryptic sites are specifically exposed in the basement membrane of angiogenic blood vessels and serve as a migration stimulus for endothelial cells (24). Exposure of cryptic sites within collagen type IV is required for angiogenesis and tumor growth \textit{in vivo}. Moreover, monoclonal antibodies specific for cryptic collagen sites (e.g., the HUIV26 cryptic epitope) generated by subtractive immunization were found to inhibit experimental metastasis \textit{in vivo} (24-26). Blocking of this cryptic site with specific antibodies also interferes with retinal angiogenesis (27). These findings suggest that ligands of cryptic binding sites in collagen may be suitable candidates to develop new strategies in the treatment of tumors as well as vascular diseases. As the exposure of cryptic sites in collagen IV is restricted to sites of angiogenesis, their binding partners may be interesting tools for the specific delivery of anticancer drugs into tumor vasculature. Therefore, the aim of our study was to use phage display technology to identify peptides that specifically bind to cryptic binding sites of MMP-2 cleaved collagen IV and to characterize their tumor-homing ability and their antiangiogenic effects.

**Results**

\textbf{Combined \textit{In vivo}/\textit{In vitro} Phage Display Screening}

Our aim was to generate peptides that specifically bind to MMP-2-processed collagen IV in tumor tissue. In a first step, we selected a pool of potential tumor-homing phage from a heptapeptide library displayed on M13 phage \textit{in vivo}. In a second step, we used this phage pool to select specific ligands to immobilized MMP-2-processed pepsin-extracted collagen IV from human placenta \textit{in vitro}. We isolated seven individual phage clones from the second round of \textit{in vitro} selection and tested their binding properties to MMP-2-processed collagen IV. One of the isolated phage clones (phage 1) displayed the heptapeptide sequence TLTYTWS and bound to MMP-2-processed collagen IV but not to native collagen IV. The other phage clones tested did not bind to collagen IV or MMP-2-processed collagen IV. None of the phage clones bound to bovine serum albumin-coated control surfaces (Fig. 1A).

\textbf{TLTYTWS Phage Specifically Binds to MMP-2-Processed Collagen IV}

To further characterize the specificity of the TLTYTWS phage, we compared its binding properties to different matrices including unprocessed collagen IV, heat-denatured collagen IV, collagen IV, which had been incubated with MMP-1, -2, -8, or -9 and MMP-2 alone. Binding properties of the TLTYTWS phage were also compared with a nonbinding control phage displaying the heptapeptide sequence SRPQITN (phage 5). As expected, the control phage did not bind effectively to any collagen matrix tested. In contrast, TLTYTWS phage effectively bound to collagen IV modified by MMP-2 but not to collagen IV incubated with other MMPs or collagen denatured by heat (Fig. 1B). These results indicate that TLTYTWS phage specifically binds to collagen IV processed by MMP-2.

**Mutation of the TLTYTWS Binding Site**

To further verify the specificity of the displayed binding motif, the binding amino acid sequence was altered, that is, phage clones were produced in which single amino acids of the binding sequence were changed to glycine (GLGYGWS, TGTYTWG, or TLTYTGS). As shown in Fig. 1C, phage particles with an altered binding sequence show impaired binding to MMP-2-processed collagen IV compared with the TLTYTWS phage. These results indicate that the sequence TLTYTWS specifically enables the phage to bind to MMP-2-modified collagen IV.

**Inhibition of TLTYTWS Phage Binding to MMP-2-Processed Collagen IV by Competition with TLTYTWS Peptide**

We next tested whether binding of the TLTYTWS phage can be blocked by the cognate peptide in a competition assay \textit{in vitro}. As shown in Fig. 2, TLTYTWS peptide blocks the binding of TLTYTWS phage in a concentration-dependent manner. Interestingly, very low amounts of peptide (10 pg/mL) were sufficient to block phage binding, suggesting high binding affinity.

**Tumor Homing of TLTYTWS Phage**

To test the tumor-homing ability, we injected TLTYTWS phage i.v. in Lewis lung carcinoma (LLC) tumor-bearing mice and compared its biodistribution with a nonbinding control phage with a modified binding motif (GLGYGWS). The output of TLTYTWS phage from tumors was ∼2 orders of magnitude higher compared with control phage (Fig. 3). In contrast, no relevant differences of TLTYTWS phage and control phage output from organs tested were observed, indicating specificity of the homing to tumors. Only in the spleen a slightly higher output of TLTYTWS phage compared with control phage was observed, which is most likely due to natural variations of the uptake of phage particles by the mononuclear phagocytic system in the spleen as reported for M13 phage (28, 29). Moreover, a nonrelated control phage displaying the peptide sequence SRPQITN, which was used as a second control, did not accumulate in tumor tissue (data not shown). To test whether tumor homing of the TLTYTWS phage is specific, we performed \textit{in vivo} competition experiments by i.v. coinjection of the cognate TLTYTWS peptide and the TLTYTWS phage in LLC tumor-bearing mice. Coinjection of the cognate peptide blocked phage homing, indicating that homing is due to the interaction of the phage with a specific target structure within the tumors (Fig. 3, inset).

**TLTYTWS Peptide Interferes with Endothelial Cell Differentiation \textit{In vitro}**

To test the influence of the TLTYTWS peptide on endothelial differentiation \textit{in vitro}, we used a tube formation assay. For quantification, branching points in the tubular network formed by human umbilical vein endothelial cells on Matrigel were counted. Interestingly, we found that TLTYTWS peptide reduced the number of branching points in a dose-dependent manner most likely due to collapse of tubular structures. Quantitatively, we found a reduction of the number of branching points of up to 60% after addition of different concentrations of TLTYTWS peptide as indicated (Fig. 4). We also
used an unrelated control peptide and found no reduction of branching points. Representative images of tubular networks in the absence or presence of TLTYTWSpeptide are shown in Fig. 4 (inset). Our data suggest that TLTYTWSpeptide blocks interactions of endothelial cells that are important to maintain the tubular network.

**TLTYTWS Peptide Inhibits Angiogenesis In vivo**

We used Matrigel plug assays to analyze the potential of TLTYTWS peptide to reduce angiogenesis in vivo (30). To quantify angiogenesis, we measured the hemoglobin content in the plugs in the presence or absence of TLTYTWS peptide (31). Accumulation of hemoglobin in the Matrigel plugs is a consequence of vessel formation and therefore is a measure of angiogenesis. We found that TLTYTWS peptide blocks angiogenesis in Matrigel plug assays in a dose-dependent manner (Fig. 5). This supports the idea that TLTYTWS peptide blocks a binding site that is functional important during angiogenesis.

**Discussion**

The aim of our study was to generate peptides binding to human collagen IV proteolytically modified by MMP-2 and to test their tumor-homing capabilities as well as their putative antiangiogenic effects. To obtain such peptides, we used a combined *in vitro*/*in vivo* phage display screening approach combining the advantages of the *in vivo* phage display (tumor specificity and low background of nonbinding phage) with the advantages of *in vitro* phage display (high target specificity). Indeed, we found a phage displaying the peptide sequence TLTYTWS that bound to human collagen IV processed by MMP-2 but neither to unprocessed collagen IV nor to collagen IV processed by other MMPs. The binding of the phage is specific, as it could be blocked by the cognate peptide in a dose-dependent manner. We further show the binding specificity of the peptide by altering the peptide sequence, which abolished specific binding. Especially, the three threonine residues in the TLTYTWS motif are vital for binding, as mutation of these amino acids yielded phages not capable of binding MMP-2-processed collagen IV.

![FIGURE 1. Binding characteristics of TLTYTWS phage *in vitro*. A. Seven individual phage clones derived from the phage display screen were tested for binding to immobilized collagen IV, collagen IV processed by MMP-2, or bovine serum albumin-coated surfaces. Phage clone 1 displayed the peptide sequence TLTYTWS and bound to MMP-2-processed collagen IV. B. Binding of TLTYTWS phage to native collagen IV, heat-denatured collagen IV, MMP-2 alone, and collagen IV incubated with MMP-1,-2,-8, or -9 was tested and compared with control phage. *, *P < 0.05 versus MMP-2-processed collagen IV. C. Recombinant phage with mutations in the displayed heptapeptide show impaired binding to MMP-2-processed collagen IV. **, *P < 0.01 versus TLTYTWS phage.](image-url)
Interestingly, the TLTYTWS phage did bind neither to heat-denatured collagen IV nor to collagen treated with MMPs other than MMP-2. These results indicate that the TLTYTWS motif binds to a cryptic binding site in collagen IV, which is specifically activated by proteolysis by MMP-2. Therefore, it is very unlikely that this peptide binds the same cryptic site that was reported by Xu et al., which is recognized by the HUIV26 antibody on thermic denaturation of collagen IV (24, 32). Interestingly, Hangai et al. recently reported that the cryptic site, which is bound by the HUIV26 antibody, is not formed in MMP-9 knockout mice but is exposed and functionally active in MMP-2 knockout mice (33). In contrast, the cryptic site binding, the TLTYTWS peptide, is exclusively generated by procession of collagen IV by MMP-2, supporting the idea that the two binding sites are not identical. Further studies are required to characterize the biochemistry of the binding site of the TLTYTWS peptide in MMP-2-processed collagen IV.

Methods influence the formation of the cryptic binding site in vitro. For comparison of binding and functional characteristics of the TLTYTWS peptide and the HUIV26 antibody during angiogenesis, it would be interesting to elucidate the binding site of the antibody, which is unknown so far. Moreover, a better understanding of the tumor homing and inhibition of angiogenesis by the TLTYTWS peptide may be provided by using tumor models of cancer of different organs or by testing the peptide in MMP-2 deficient mice.

As cryptic binding sites in collagen IV have been shown to be present during tumor angiogenesis in vivo, we tested whether TLTYTWS phage shows tumor-homing capabilities. Indeed, we found TLTYTWS phage accumulation in xenograft tumors. These results support the idea that the phage binds a cryptic binding site that is formed only by proteolytic degradation of collagen IV during tumor angiogenesis. TLTYTWS phage homing to tumors can be blocked by coinjection of the cognate peptide, supporting the idea that TLTYTWS binds a specific target structure within tumors. Specificity of tumor homing of the TLTYTWS phage is also shown by our finding that point mutations in the peptide sequence preclude the accumulation in tumor tissue. Our results suggest that the peptide can potentially be used as a conjugate for anticancer drugs facilitating specific accumulation in tumors.

It is well documented that ligands of cryptic binding sites in collagen IV block tumor angiogenesis as well as pathologic blood vessel formation, for example, in diabetic retinopathy (14, 27). Therefore, it is not surprising that TLTYTWS peptide also interferes with angiogenesis. In vitro, we found that TLTYTWS peptide significantly reduces the number of branching points in an endothelial cell differentiation assay. This indicates that blockage of the interaction of the cryptic binding site with its presumed natural ligand by an excess of TLTYTWS peptide reduces the density of the network but does not completely block differentiation in this system. The idea that TLTYTWS peptide blocks a functionally relevant interaction during angiogenesis is further corroborated by the finding that it inhibits angiogenesis in vivo as shown in Matrigel plug assays.
Taken together, our data indicate that a cryptic site binding, the TLTYTWS motif, is formed on proteolytic degradation of collagen IV by MMP-2 during angiogenesis. This novel site is functionally relevant in angiogenesis and may potentially be an appropriate target structure for new selective tumor therapies. In the future, a variety of cryptic sites in the extracellular matrix may be found modulating angiogenesis and vascular differentiation.

Materials and Methods

Materials

Human MMPs were purchased from Calbiochem. The M13 phage library, the M13KE vector DNA, the enzymes Acc65I and Eagl, and the phage-host-bacteria Escherichia coli ER2738 were from New England Biolabs. Oligonucleotides were from MWG Biotech. Peptide synthesis was done by GL Biochem. Xylazine was from Bayer. Ketamine was from Pfizer. Dulbecco’s PBS (DPBS) was purchased from Invitrogen. Collagen IV from human placenta was purchased from Sigma-Aldrich. The collagen IV was pepsin-extracted and acid-soluble. For the experiments, the human collagen IV was dissolved at 1 mg/mL in 0.5 mol/L acetic acid according to the manufacturer’s instructions. According to the manufacturer’s product quality control, this collagen is cleaved by collagenases. All other chemicals were purchased from Sigma-Aldrich.

Cell Culture

Murine LCC cells were purchased from Cell Lines Service and maintained in DMEM (stable glutamine; Biochrom) containing 10% FCS, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Human umbilical vein endothelial cells (PromoCell) were cultured in tissue culture flasks (Greiner) coated with 1 mg/mL collagen G (Biochrom) in endothelial cell growth medium (PromoCell) at 37°C in a humidified atmosphere containing 5% CO₂. Human umbilical vein endothelial cells were used at passages 1 to 3.

Animals

All animals were purchased from Charles River and kept at our animal care facility for 2 weeks before use. All animal experiments were carried out in accordance with the guidelines for the use of living animals in scientific studies and the German law for the protection of animals. The animal experiments were approved and supervised by the Government...
of Upper Bavaria (Regierung von Oberbayern; record no. 55.2.1-54-2531-34-02 and 55.2-1-54-2531-52-07).

LCC Cell Tumor Model

Murine LLC cells were injected s.c. in the right femoral region of 8-week-old female NMRI nude mice (1 × 10^7 LLC in a total volume of 100 μL in DPBS under ether anesthesia). After 14 days, mice were used for experiments. The vascularization of the LLC tumors under these conditions was analyzed by others (34, 35).

In vivo/In vitro Phage Display Screening

An unselected M13-Ph.D.-7 phage display peptide library was injected in the tail vein of a LLC tumor-bearing mouse, which was previously anesthetized by an i.p. application of 16 mg/kg xylazine and 100 mg/kg ketamine. Ten minutes after application of the library, the mouse was perfused with DPBS and the tumor was explanted. The tumor was homogenized with a Dounce homogenizator in DPBS. The homogenate was centrifuged at 2,700 × g for 5 min and the pellet was washed four times with DPBS. Residual phages were eluted with 900 μL of an E. coli ER2738 suspension at 37°C for 30 min in an incubator. Ten microliters of this suspension were used for the determination of the phage titer. The residual volume was used for phage amplification in 20 mL E. coli ER2738 suspension according to the manufacturer’s instructions. This solution is called the phage pool of this selection round.

The phage titer was determined according to the manufacturer’s instruction with the exception that X-gal was replaced by S-gal and ferric ammonium citrate (both Sigma) according to the manufacturer’s instruction. Then, 96-well microtiter plates (Greiner) were coated with 10 μg pepsin-extracted human collagen IV or 10 μg bovine serum albumin per well for 1 h at room temperature. Wells were then washed three times with DPBS/Tween 20 (0.05%), blocked with bovine serum albumin (1%)/DPBS/Tween 20 (0.05%) for 1 h at room temperature, and washed again and wells that were not incubated with MMPs were filled with 200 μL H2O. For proteolytic degradation of collagen IV, wells were incubated with 260 ng active human MMP-1, -2, -8, or -9, respectively. The wells were washed with 30 volumes of DPBS/Tween 20 (0.2%). For competition experiments, coated wells were preincubated with 1 μg to 0.1 μg of TLTYTWSGGKCC peptide in DPBS for 1 h at room temperature on a shaker and the supernatant was removed. Then, 1 × 10^11 pfu of the amplified phages were incubated with DPBS/Tween 20 (0.2%) in the coated wells for 1 h at room temperature on a shaker. After washing with 10 volumes of DPBS/Tween 20 (0.2%), the bound phages were eluted by adding E. coli ER2738 bacteria as described above. Ten microliters of the bacteria/phage suspension were used for phage titer determination as described above.

Phage Binding Assay

Wells were coated with 10 μg/well unmodified pepsin-extracted human collagen IV or with 10 μg/well pepsin-extracted human collagen IV, which was heat denatured at 99°C for 6 min in 100 μL DPBS or 260 ng/well recombinant human MMP-2 as described above. For proteolytic procession of pepsin-extracted human collagen IV, the wells were incubated at 37°C for 20 h with 260 ng active human MMP-1, -2, -8, or -9, respectively. The wells were washed with 30 volumes of DPBS/Tween 20 (0.2%). For competition experiments, coated wells were preincubated with 1 μg to 0.1 μg of TLTYTWSGGKCC peptide in DPBS for 1 h at room temperature on a shaker and the supernatant was removed. Then, 1 × 10^11 pfu of the amplified phages were incubated with DPBS/Tween 20 (0.2%) in the coated wells for 1 h at room temperature on a shaker. After washing with 10 volumes of DPBS/Tween 20 (0.2%), the bound phages were eluted by adding E. coli ER2738 bacteria as described above. Ten microliters of the bacteria/phage suspension were used for phage titer determination as described above.

Molecular Cloning of Phage Binding Motifs

The modification of the phage binding motif was done by standard cloning procedures. The corresponding oligonucleotides mut1_for and mut1_rev, mut3_for and mut3_rev, and mut4_for and mut4_rev were annealed (Table 1). The M13KE phage DNA was used as vector after restriction endonuclease cleavage with 10 units Acc65I/μg DNA and 10 units EagI/μg DNA and ligated with the annealed oligonucleotides using T4 ligase (Fermentas) according to the manufacturer’s instructions. E. coli ER2738 were transformed with the inactivated ligation reaction using the TransformAid bacterial transformation kit (Fermentas) according to the manufacturer’s instructions. The

Table 1. Used Oligonucleotides and Their Nucleotide Sequence

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<th>Oligonucleotide</th>
<th>Oligonucleotide Sequence</th>
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<tr>
<td>mut1_for</td>
<td>5'-GTACCTTTTCTATTCTCCTGTCAGTATGGTTAGGTTGCTGCTTGAGGTGTC-3'</td>
</tr>
<tr>
<td>mut1_rev</td>
<td>5'-GTACCTTTTCTATTCTCCTGTCAGTATGGTTAGGTTGCTGCTTGAGGTGTC-3'</td>
</tr>
<tr>
<td>mut3_for</td>
<td>5'-GGCGAGAAGCTCCAGACGACATACGATACGGAGTTAGGTTAGGTTGAGGTGTC-3'</td>
</tr>
<tr>
<td>mut3_rev</td>
<td>5'-GGCGAGAAGCTCCAGACGACATACGATACGGAGTTAGGTTAGGTTGAGGTGTC-3'</td>
</tr>
<tr>
<td>mut4_for</td>
<td>5'-GGCGAGAAGCTCCAGACGACATACGATACGGAGTTAGGTTAGGTTGAGGTGTC-3'</td>
</tr>
<tr>
<td>mut4_rev</td>
<td>5'-GGCGAGAAGCTCCAGACGACATACGATACGGAGTTAGGTTAGGTTGAGGTGTC-3'</td>
</tr>
<tr>
<td>mut_for</td>
<td>5'-GCCTCTTTTTGGAGGCTTTTT-3'</td>
</tr>
<tr>
<td>mut_rev</td>
<td>5'-ATTTGACCATCATCTACGTGCTGAGGTGTC-3'</td>
</tr>
<tr>
<td>−96Gffl</td>
<td>5'-CCTTATGAGACGAGAGCCCACTCTACGTGCTGAGGTGTC-3'</td>
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transformed bacteria were plated with 3 mL top agarose on LB/S-gal/IPTG plates and incubated overnight at 37°C. Resulting phages were amplified as described above. The amplified phages were added to an E. coli ER2738 suspension and cultivated at 37°C overnight in a shaker. The double-stranded DNA of the phage was obtained by a standard plasmid DNA extraction using the E.Z.N.A. plasmid miniprep kit I (peqLab). The inserted DNA was amplified by standard PCR procedures using the oligonucleotides mut_for and mut_rev (Table 1) and used for DNA sequencing with the oligonucleotide -96GIII as sequencing primer (Table 1). DNA sequencing was done at MWG Biotech. Phages with the correct sequence were amplified and tested as described above.

**Distribution of Phage In vivo**

Then, 1 × 10^{11} pfu of the TLTYTWS phage, a control phage (SRPQITN), or a phage with a modified binding motif (GLGYGWS), as described above, were injected i.v. in three LLC tumor-bearing mice per phage after an i.p. application of the anesthetics xyazine (16 mg/kg) and ketamine (100 mg/kg). All experiments were done in triplicates and repeated three times. After 10 min, the mice were perfused with DPBS and brain, muscle, stomach, kidneys, heart, lung, spleen, liver, and tumor were explanted and weighed. Tissues were homogenized with an Ultra-Turrax T25 homogenizer (IKA Labortechnik) in LB medium. Phage was eluted by the addition of E. coli ER2738 bacteria and the phage titer was determined as described above. To determine the pfu of the injected phage, 1 × 10^{11} pfu of the used phage were aspirated in a syringe and the resulting phage titer was determined as described above.

For in vivo competition of phage homing to LLC tumor-bearing mice, 150 μL TLTTYWGGGGKK peptide and 1 × 10^{11} pfu of the TLTYTWS phage were coinjected i.v. in three LLC tumor-bearing mice per experiment after an i.p. application of the anesthetics xyazine (16 mg/kg) and ketamine (100 mg/kg). After 10 min, the mice were perfused with DPBS and tumors and explants were weighed. Tissues were homogenized with an Ultra-Turrax T25 homogenizer (IKA Labortechnik) in LB medium. Phage was eluted by the addition of E. coli ER2738 bacteria and the phage titer was determined as described above.

**In vivo Matrigel Plug Assay**

Then, 200 ng basic FGF (Immunotoools) with or without the addition of TLTTYWGGGGKK peptide were mixed with 500 μL growth factor-reduced Matrigel (BD Bioscience) and injected s.c. in the abdomen of female 18-week-old CD1 mice. After 11 days, mice were sacrificed and the Matrigel plugs were explanted and weighed. The Matrigel plugs were homogenized in a volume of 500 μL DPBS by ultrasound. As a measure for angiogenesis, the hemoglobin content of the plugs was used as determined by the Drabkin’s assay (36) according to the manufacturer’s instructions (31). As a control, 200 ng basic FGF and 10 μg of the unrelated SRPQITNGGGKK peptide was mixed with 500 μL growth factor-reduced Matrigel, injected, and processed as described above.

**Endothelial Cell Differentiation Assay**

We performed a tube formation assay to test the ability of the TLTTYW peptide to inhibit angiogenesis in vitro as described previously (37). Then, 24-well plates (Greiner) were coated with 100 μL/well growth factor-reduced Matrigel (10 μg protein/mL) for 1 h at 37°C. Then, 5 × 10^{10} human umbilical vein endothelial cells were seeded per well in a volume of 1 mL endothelial cell growth medium containing 1 ng/mL basic FGF. TLTTYW peptide and a unrelated control peptide were added to the medium in different concentrations (0.1, 1, and 10 μg/mL). No peptide was added to control wells. All test samples were done as triplicates. After 16 h at 37°C, images were captured using a microscope with a CCD camera (Carl Zeiss). As a measure for angiogenesis, the branching points of the formed tubular network per field of view were counted.

**Statistical Analysis**

Results are reported as arithmetic mean ± SEM. For analyzing the results, a t test based on range (38) was used. In general, P < 0.05 was regarded as significant.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Prof. Dr. R. Senekowitsch-Schmidke (Department of Nuclear Medicine, Technischen Universität München) for support.

**References**

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*Mol Cancer Res* Published OnlineFirst July 7, 2009.