Lymphatic Zip Codes in Premalignant Lesions and Tumors

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Abstract

Blood vessels in tumors are morphologically and functionally distinct from normal resting blood vessels. We probed lymphatic vessels in premalignant lesions and tumors by in vivo screening of phage-displayed peptide libraries, asking whether they too have distinctive signatures. The resulting peptides begin to define such signatures. One peptide identified the lymphatics in a human melanoma xenograft. Another recognized the lymphatics in prostate cancers but not in premalignant prostate lesions; this peptide similarly identifies human prostate cancer lymphatics. A third was selective for the lymphatics in the premalignant prostate lesions. A fourth identified the lymphatics in dysplasias and squamous carcinomas of the cervix and skin. None recognize lymphatics in normal tissues. Thus, tumor development is associated with organ- and stage-specific changes in lymphatics. Systemic treatment of mice with fusions of a lymphatic homing peptide and a proapoptotic motif reduced the number of tumor lymphatics in prostate tumor and melanoma, forecasting future lymphatic targeting agents for detection and therapeutic intervention. (Cancer Res 2006; 66(11): 5696-706)

Introduction

The endothelial lining of blood vessels is highly diversified. Many, and perhaps all, normal tissues impart a tissue-specific "signature" on their vasculature, and tumor vessels differ from normal vessels both in morphology and molecular composition (1). Tumors induce angiogenesis to support expansive growth (2) and many of the changes in tumor vessels are angiogenesis related (3–6). Moreover, tumor blood vessels have tumor type–specific and, in some stages, stage-specific characteristics; in vivo screening of phage libraries has yielded distinct sets of homing peptides selectively recognizing angiogenic signatures in two transgenic mouse models of organ-specific tumorigenesis. Homing peptides can also distinguish the angiogenic blood vessels of premalignant lesions from those of fully malignant lesions in the same tumor model (7, 8), indicating that vascular changes mirror the stage of tumor development.

The lymphatic system constitutes a second vascular system, one that has only an efferent arm. Tumors frequently induce lymphangiogenesis, as well as co-opt existing lymphatics (9–11). Tumors may contain intratumoral lymphatics, but, more commonly, an extensive network of lymphatic vessels is present around tumor tissue (12–14). The lymphatics within tumors, when present, are generally nonfunctional in fluid transport (14), possibly reflecting compression by interstitial pressure and blockage by intratumoral tumor cells. The lymphatic vessels in and around tumors are an important conduit of metastasis. Indeed, growth factor–stimulated enhancement of lymphatic vessel expression in tumors increases metastasis (15, 16). Conversely, inhibiting lymphangiogenesis suppresses lymphatic metastasis, but generally does not affect tumor growth (17).

A peptide that selectively binds to the endothelial cells of lymphatics associated with a xenotransplanted human breast tumor has been described (13). This was the first demonstration that tumor lymphatics can differ from normal lymphatics, but the larger question of whether tumor lymphatics are generally distinguishable from normal lymphatics has been unanswered.

Here, we identify homing peptides that specifically recognize tumor lymphatics or lymphatics in premalignant lesions in a set of distinctive organ-specific tumor models in mice. Our results show that tumor lymphatics, like tumor blood vessels, express specific markers, and that these lymphatic markers are tumor type specific and distinct from blood vessel markers in the same tumors. The tumor-specific lymphatic vessel markers may be useful in early detection and tumor targeting.

Materials and Methods

Cell Lines, Mice, and Tumors

The following cell lines were maintained in DMEM supplemented with 10% FCS: C8161 human melanoma, MDA-MB-435 human breast cancer, KRIB human osteosarcoma, and human prostate cancer cells PPC1 and DU145. LNCaP human prostate cancer cell line was grown in RPMI 1640 with 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, and 1.5 g/L sodium bicarbonate supplemented with 10% FCS. M12 human prostate cancer cell line was cultured in RPMI 1640 with 5 μg/mL insulin-transferrin-selenium, 2.5 μg/mL fungizone, 50 μg/mL gentamicin, 0.2 μmol/L dexamethasone, 10 ng/mL epidermal growth factor, and 5% FCS (18). To produce tumors, nude BALB/c and C56BL/6 mice were s.c. (C8161, KRIB, and PPC1) or orthotopically (MDA-MB-453, PPC1, DU145, M12, and LNCaP) injected with 1 × 106 tumor cells. Transgenic mouse tumor models included transgenic adenocarcinoma of the mouse prostate (TRAMP), mouse mammary tumor virus (MMTV)-PyMT breast cancer, and K14-HPV16 cervical cancer. To initiate cervical carcinogenesis, female K14-HPV16 mice (19) were treated with 17β-estradiol (E2; refs. 20, 21). Briefly, 1-month-old virgin female transgenic (heterozygous K14-HPV16, 1203#1) and nontransgenic (FVB/n) mice were anesthetized with isoflurane, and continuous release pellets that deliver E2 at doses of 0.05 mg over 60 days (Innovative...
Tissues were embedded in Tissue-Tek OCT (Tissue-Tek, Elkhart, IN) and the mice were perfused with 4% PFA 30 minutes after the injection of phage. Each mouse and the phage were rescued and tittered. For histology analysis, phage. After 7 minutes, the mice were perfused through the heart with PBS amplified in sheep anti-rat IgG Dynabeads (M450; Dynal, Oslo, Norway). Phage that albumin (BSA). Podoplanin-positive cells were then isolated using M450 at 4
unbound phage, incubated with rat anti-mouse podoplanin for 45 minutes prostate tissue or prostate tumor. The cells were washed to remove
prostate, second, antipodoplanin magnetic beads were used to isolate lymphatic endothelial cells. We did two to three rounds of ex vivo selection and two to three rounds of in vivo selections.

For the ex vivo selections, cell suspensions were prepared from normal prostate of tumor-free littermates of TRAMP mice, premalignant prostate of 14- to 16-week-old TRAMP mice, and tumor tissues of 25- to 28-week-old TRAMP mice. The C57BL6 TRAMP mice display mild to severe hyperplasia by the time they are 12 weeks of age, and severe hyperplasia has developed by 18 weeks (23, 24). Thus, the premalignant lesions of prostate we used represent a mixture of mild or severe hyperplasia. Collagenase IA (1 mg/mL; Sigma, St. Louis, MO) was used to disperse the tissues. About 1 × 107 normal prostate cells were incubated at 4°C for 3 hours with 5 × 1015 plaque-forming units (pfu) of T7 phage displaying a C7X7C peptide library. The samples were centrifuged at 1,200 rpm for 10 minutes; the supernatant (the normal prostate-subtracted phage library) was recovered and then incubated overnight at 4°C with 5 × 107 cells derived from premalignant prostate tissue or prostate tumor. The cells were washed to remove unbound phage, incubated with rat anti-mouse podoplanin for 45 minutes at 4°C, and washed thrice with cold PBS containing 0.5% bovine serum albumin (BSA). Podoplanin-positive cells were then isolated using M450 sheep anti-rat IgG Dynabeads (M450; Dynal, Oslo, Norway). Phage that bound to the podoplanin-positive cell population were rescued and amplified in Escherichia coli. In vivo phage library screening was done as described (13).

Homing Specificity of Phage

In vivo homing specificity of phage was tested as described (22). Briefly, mice bearing tumors were anesthetized and i.v. injected with 1–5 × 1012 pfu phage. After 7 minutes, the mice were perfused through the heart with PBS containing 0.5% BSA. The tumor and control organs were dissected from each mouse and the phage were rescued and littered. For histology analysis, the mice were perfused with 4% PFA 30 minutes after the injection of phage. Tissues were embedded in Tissue-Tek OCT (Tissue-Tek, Elkhart, IN) and 5 μm sections were prepared for phage immunostaining.

Antibodies and Immunohistology

Custom immunization to produce a rabbit antiserum against mouse Prox-1 was done by ProteinTech, Inc. (Chicago, IL) New Zealand White rabbits were immunized with a fusion protein of glutathione S-transferase (GST)–C9C9C-terminal fragment of Prox-1 protein. The antibody was affinity purified on the fusion protein and absorbed with GST. The resulting antibody preparation (1.8 mg/mL) gave a titer of 1:10,000 against the fusion protein in ELISA. Immunofluorescence staining of tissue sections with the anti-Prox-1 antibody gave a pattern of nuclear staining. Antibodies against the lymphatic markers anti-LYVE-1 (13) and anti-podoplanin [kindly provided by T. Petrova and K. Alitalo (Biomedicum, University of Helsinki, Helsinki, Finland)], rat monoclonal anti-mouse CD31 (BD PharMingen, San Diego, CA), rat anti-mouse MECA-32 (BD PharMingen), rabbit polyclonal anti-T7 phage, rabbit anti-mouse cleaved caspase-3 (ASP175; Cell Signaling Technology, Danvers, MA), and rat anti-mouse vascular endothelial growth factor receptor 3 [VEGFR3 provided by H. Kubo and K. Alitalo (Biomedicum, University of Helsinki)] were used for immunohistochemical staining of frozen tissue sections as described (8, 13).

The corresponding secondary antibodies were added and incubated for 1 hour at room temperature: AlexaFluor-488 goat anti-rabbit or rabbit IgG (1:1,000; Molecular Probes, Eugene, OR), AlexaFluor-594 goat anti-rat or rabbit IgG (1:1,000; Molecular Probes), AlexaFluor-594 donkey anti-mouse or goat IgG (1:1,000; Molecular Probes), and AlexaFluor-488 donkey anti-mouse or goat IgG (1:1,000; Molecular Probes). The slides were washed thrice with PBS and mounted in Vectashield Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Blood vessels were also visualized by i.v. injecting Lycopersicon esculentum (tomato) lectin conjugated to fluorescein (100 μg of lectin in 200 μL PBS; Vector Laboratories).

Tissue distribution of fluorescein-labeled peptides (25) was studied by i.v. injecting the peptide (100-150 μg in 200 μL PBS) into the mice. The injected peptides were allowed to circulate 30 minutes to 2 hours, and the mice were perfused with 4% paraformaldehyde through the left ventricle of heart. Tissues were dissected and frozen in OCT embedding medium (Tissue-Tek). The frozen sections were prepared for immunohistologic analysis.

Peptide Synthesis

Peptides were synthesized in our peptide facility using Fmoc chemistry in a solid-phase synthesizer. The peptides were purified by high-performance liquid chromatography and confirmed by mass spectrometry. Fluorescein-conjugated peptides were synthesized as described (25). The LSD and REA peptides were synthesized as the chimera with the proapoptotic motif D(KLAKLAK)2, (26).

Targeted Proapoptotic Peptide Treatment of Tumor-bearing Mice

Prostate cancer model. Orthotopic xenografted prostate tumors were established by injecting 1 × 106 PPC1 human prostate cancer cells into the mouse prostate. Fifteen days postinoculation, the mice were i.v. injected with D(KLAKLAK)2-CREAGRKAC, or PBS. Mice were given 200 μg of the conjugate per week divided into two injections (26, 27).

Melanoma model. Nude BALB/c mice were s.c. injected with 1 × 106 C8161 human melanoma cells. Treatment started when mean tumor volumes reached ~100 mm3. Mice with size-matched tumors were randomized into four groups. The therapeutic group received a chimera of tumor-homing peptide with the proapoptotic motif D(KLAKLAK)2-CLSDGKRKC, or PBS. The tumor-bearing mice were i.v. injected with 200 μg/dose/mouse weekly for 3 weeks (26, 27).

The mice were monitored for weight loss, and tumors were dissected and weighed at the termination of the experiment. Histologic analysis was done to evaluate the density of tumor lymphatics and blood vessels. Apoptotic lymphatic endothelial cells were visualized by double staining with anticleaved caspase-3 and antipodoplanin antibodies. The animal experiments reported here were approved by the Animal Research Committee of Burnham Institute for Medical Research.

Phage Overlay of Tissue Sections From Human Cancer

The frozen sections of human prostate tumor specimens were obtained from Dr. Daniel Mercola (Sidney Kimmel Cancer Center, La Jolla, CA). The sections (5 μm) were preincubated with blocking buffer (5% normal goat serum and 0.5% BSA in 1× PBS) for 1 hour at room temperature, washed thrice with diluted blocking buffer (1:10), and phage (3 × 1010 pfu) were incubated on the section for 4 hours. After three washes, rabbit anti-phage antibody (10 μg/mL) was added and the phage incubated for 2 hours. The slides were washed and incubated with AlexaFluor-488 goat anti-rabbit IgG for 1 hour. After further washes, the slides were mounted with Vectorshield (Vector Laboratories).

Statistical Analysis

Student’s t test was used in statistical analysis of the results. The bar diagrams show mean and SD.

www.aacrjournals.org 5697 Cancer Res 2006; 66: (11). June 1, 2006

Lymphatic Zip Codes in Premalignant Lesions and Tumors

Research of America, Sarasota, FL) were implanted s.c. in the dorsal back skin. Subsequent pellets were implanted at 3 and 5 months of age for a total of 6 months of hormone treatment. K14-HPV16 mice were maintained in the FVB/n background (FVB/n; The Jackson Laboratory, Bar Harbor, ME). The mice were maintained in accordance with the University of California, San Francisco, institutional guidelines governing the care of laboratory mice. The animal experimentation was approved by Animal Research Committees at University of California, San Francisco, or Burnham Institute for Medical Research.
Results

Phage targeting of lymphatics in C8161 melanoma. We chose the C8161 human melanoma as the first topic because xenografts of tumors generated with this cell line in nude mice contain lymphatic vessels that are not recognized by the homing peptide LyP-1, which binds to lymphatic endothelial cells in breast carcinomas (13). Our experimental design was aimed to determine whether lymphatic homing peptides having analogous specificity for the melanoma-associated lymphatics could be identified. We modified our earlier protocols to increase the probability of obtaining peptides that recognize tumor lymphatics. We incubated a phage display library with a cell suspension of whole C8161 tumor tissue, allowing phage to bind, and then used immunomagnetic beads to isolate lymphatic endothelial cells that carried along any phage bound to these cells. This enrichment step yielded a phage pool that bound 250-fold more efficiently to the isolated cells than nonrecombinant phage (Supplementary Fig. S1A). The enriched phage pool was used in subsequent in vivo rounds to select phage that homed to C8161 xenograft tumors. Two rounds of selection in vivo produced a 40-fold enrichment of phage (Supplementary Fig. S1B). There was no enrichment in the several control organs tested.

The 48 phage clones from the second in vivo round of phage pool selection included five clones that appeared most frequently, and these were analyzed further. Two clones displaying peptides with related amino acid sequences (CLSDGKRKC and CLDGGRPKC) bound to cell suspensions prepared from C8161 tumors; the stronger binder, CLSDGKRKC, bound 100-fold more than control phage. I.v. injection of phage into nude mice bearing C8161 tumors showed that both phage homed selectively to the tumors; CLSDGKRKC was about twice as efficient as CLDGGRPKC (the results for CLSDGKRKC are shown in Fig. 1A). The CLSDGKRKC peptide (referred to below as LSD) was chosen for further study.

To establish that the homing ability of LSD phage is due to the displayed peptide sequence, we chemically synthesized the peptide as a fluorescein conjugate peptide and i.v. injected the conjugate into C8161 tumor mice. After 2 hours of circulation, the peptide was detected within the tumors (Fig. 1B), but not in control organs (Supplementary Fig. S1C). Staining of tissue sections with the lymphatic vessel markers podoplanin, Prox-1, LYVE-1, and VEGFR3 showed colocalization of the LSD fluorescence with them (Fig. 1C), whereas there was no colocalization with the blood vessel markers MECA-32 and CD31 (Fig. 1D). Quantification showed that 85% of the lymphatic vessels that were positive for the peptide were also positive for podoplanin.

We further tested the homing of LSD phage to other types of cancer, including the MDA-MB-435 human breast cancer xenografts recognized by the previously described lymphatic homing peptide, LyP-1 (13). I.v. injected LSD phage did not appreciably home to MDA-MB-435 tumors (see below). These data show that LSD-peptide selectively homes to the lymphatic vessels in C8161 melanoma.
Phage targeting of lymphatics in premalignant lesions and tumors of prostate. Seeking to further generalize the proposition that tumor-associated lymphatics might have organ-specific signatures, we selected lymphatic homing peptides in the TRAMP transgenic mouse model of de novo prostate carcinogenesis (28). Immunohistochemical analysis had revealed abundant lymphatics associated with premalignant lesions and tumors in this model (Supplementary Fig. S2A). As it is possible to access premalignant lesions in this system, we also explored the possibility of distinguishing the lymphatics of such lesions from those of fully developed tumors. We studied TRAMP mice inbred into C57BL6, a genetic background wherein prostate tumorigenesis occurs over a 30-week time course to terminal disease, with a discernable premalignant phase (~10-20 weeks).

To isolate peptides that selectively home to fully developed tumors in the TRAMP model, we first pretreated the phage library with cell suspensions derived from normal prostate to decrease the abundance of phage that bind to normal prostate. The normal prostate-subtracted library was then enriched by two rounds of ex vivo selection on lymphatic endothelial cells immunopurified from tumors of 25- to 28-week-old TRAMP mice. Three subsequent in vivo selection rounds yielded a phage pool that showed nearly 50-fold enrichment for tumor homing. Five peptide sequences were represented more than once in this pool. Three of these phage clones with amino acid sequences CREAGRKAC, CSMSAKKKC, and CKTRVSCGV showed robust binding to tumor-derived cell suspensions and were further tested in vivo. I.v. injected CREAGRKAC phage became 50-fold enriched in TRAMP tumors relative to nonrecombinant phage, whereas the other two phage showed ~30-fold enrichment. We chose the CREAGRKAC (REA) for further study.

To screen for peptides recognizing the premalignant lymphatics, we first treated the phage library with cell suspensions derived from normal prostate, and the subtracted library was then enriched on immunopurified lymphatic endothelial cell suspensions derived from prostates containing premalignant lesions, the so-called prostatic intraepithelial neoplasia, or PIN (in 14- to 16-week-old mice). The sequential ex vivo selections yielded a phage pool that was 60-fold enriched for binding to the target cells, and a 30-fold enrichment for homing to prostate with PIN lesions was obtained in a subsequent in vivo selection. Five phage clones were chosen for evaluation of in vivo homing based on their frequent appearance among 64 clones sequenced (32 clones each from the second ex vivo round and the third in vivo round). Of these, three clones with amino acid sequences CAGRRSAYC, CASLSCR, and CSGGKVLDC, bound to cell suspension derived from PIN lesions (data not shown). These candidates were further tested in vivo individually. Phage-displayed peptides CAGRRSAYC, CSGGKVLDC, and CASLSCR showed 24-, 14-, and 12-fold enrichment to PIN lesions relative to nonrecombinant phage, respectively. The CAGRRSAYC (AGR) was chosen for further study.

To evaluate the specificity of the REA and AGR peptides, we i.v. injected the phage into TRAMP mice with either premalignant PIN lesions or prostate tumors, or into their tumor-free (transgene negative) male littermates with normal prostates. The results showed that the REA phage homes to tumors, but not to PIN lesions or normal prostate, whereas the AGR phage homes only to PIN (Fig. 2A). Neither phage was found in other tissues, including lymph nodes, kidneys, lungs, skin, or intestine, at levels higher than the nonrecombinant control phage.

In vivo distribution of fluorescein-conjugated REA and AGR peptides after i.v. injection confirmed the phage results. The REA peptide accumulated in prostate tumors, showing 90% overlap with podoplanin-positive lymphatic vessels, whereas PIN lesions, normal prostate (Fig. 2B), and control organs (Supplementary Fig. S2B) were negative. The AGR peptide selectively homed to PIN lesions, but little or no peptide was seen in prostate tumors, normal prostate tissue (Fig. 2B), or in control tissues (Supplementary Fig. S2C).

To study the association of REA and AGR peptides with the vasculature, the phage or fluorescein-labeled peptides were i.v. injected into TRAMP mice and phage and peptide localization was compared with lymphatic and blood vessel markers localized with antibodies. The phage and their cognate peptides each showed substantial colocalization with the lymphatic markers podoplanin, VEGFR3, LYVE-1, and Prox-1 in their respective lesions, whereas their localization was entirely distinct from that of the blood vessel markers CD31 and MEC-32. The overlap of the peptides with Prox-1 was less obvious than with the other markers, presumably because Prox-1 is nuclear, whereas the peptides associate with the cell membrane. The results for the REA and AGR peptides are shown in Fig. 2C and D, and for the REA phage in Supplementary Fig. S2D.

Homing peptide for lymphatic vessels in cervical cancer. In a previous study from our laboratories, we identified a homing peptide for dysplastic skin lesions in K14-HPV16 transgenic mice, which develop skin cancers (7). This peptide, CNRRTKAGC, is similar to LyP-1 (CGNKRTRGC), which selectively recognizes lymphatic vessels and tumor cells in breast cancers (13). Because of this similarity, we asked whether the CNRRTKAGC peptide (LyP-2) also recognizes tumor lymphatics. We tested the LyP-1 and LyP-2 peptides in skin and cervical cancers of the K14-HPV16 transgenic mice. In addition to spontaneously developing angio- genic dysplasias and then squamous cell carcinomas of the skin (29), female K14-HPV16 mice develop cervical cancers when their normal cyclic estrogen levels are sustained with time release pellets (20). The estrogen-treated females undergo neoplastic progression in the cervix mimicking, that inferred for human cervical carcinogenesis (20, 21, 29). The premalignant cervical lesions (also called cervical intraepithelial neoplasia, CIN) and cervical tumors of these mice contain abundant lymphatic vessels as detected by immunostaining for lymphatic markers (Supplementary Fig. S3A).

I.v. injected LyP-2 phage showed robust homing both to the premalignant and malignant lesions in the cervix, but not to normal cervix (Fig. 3A). Fluorescein-labeled LyP-2 peptide also accumulated in the cervical lesions, colocalizing with LYVE-1 (Fig. 3B, top) and podoplanin (82% overlap; data not shown), but not with MEC-32 (Fig. 3B, bottom). Additionally, occasional foci of scattered cells in the stroma were labeled, with some apparent intracellular localization; the identity of these cells is currently unresolved. No peptide accumulation was observed in normal cervix (Fig. 3B) or in other control tissues, either in lymphatics or in nonvascular cells (Supplementary Fig. S3B). LyP-2 also homed to the lymphatics associated with dysplasias and squamous cell carcinomas of the skin in male and female K14-HPV16 mice, but not to normal skin lymphatics (data not shown).

Specificity of lymphatic homing peptides for different types of tumors. Having isolated phage-displayed peptides that homed to the lymphatics of melanoma, prostate, or cervix (the origin and specificity of these peptides is summarized in Table 1), we asked whether they recognized common determinants of the
tumor-associated lymphatic vasculature or organ/tumor selective signatures. The lymphatic homing peptides derived from the different tumor models were tested for their ability to recognize the lymphatics of other tumors. I.v. injected LSD phage did not home to xenotransplant tumors derived from the MDA-MB-435 breast tumor cell line (Fig. 4A, left). This phage also did not appreciably home to transgenic mouse tumors of the breast or prostate, or to PPC1 human prostate cancer xenografts; possible low-level homing was seen to squamous carcinomas of the skin in K14-HPV16 mice, and to KRIB human osteosarcoma xenografts.

In vivo injection of fluorescein-labeled LSD peptide, followed by histologic analysis of peptide distribution, agreed well with the phage results. As shown in Fig. 4A (right), strong LSD peptide fluorescence was seen in the C8161-derived tumors, the model in which the peptide was selected. The C8161 tumors were positive in nude mice representing two different genetic backgrounds (BALB/c and C57BL/6; shown for the BALB/c strain in Fig. 4A, right). In agreement with the phage data, KRIB tumors were weakly positive with the fluorescent peptide, and the other tumors, including the skin cancers, were negative. These results show that the LSD peptide selectively recognizes the lymphatics in the C8161 melanoma-derived tumors.

To profile the homing peptide specificity of the AGR peptide in different types of premalignant lesions, we used three transgenic mouse models, TRAMP, K14-HPV16/E2, and MMTV-PyMT, which, respectively, develop prostate, cervical, or breast neoplasias that subsequently progress to overt cancer. Both AGR phage (Figs. 2A and 4B) and fluorescent peptide (Fig. 2B and D) showed marked preference for the PIN lesions in TRAMP mice; there was little homing of the phage and no detectable homing of the peptide to similar premalignant lesions or malignant tumors in the other two models (Fig. 4B and Supplementary Fig. S2G).

The REA phage, which was identified in the TRAMP model, also homed to xenografts obtained by orthotopically inoculating into nude mice cells from the human prostate cancer cell lines PPC1, M12, DU145, and LNCaP (Fig. 4C). These xenografted tumors were also positive with the fluorescein-conjugated REA peptide (the results for PPC1 are shown in Fig. 4D). In contrast, the MDA-MB-435, C8161, and KRIB xenografts, as well as the de novo breast and skin cancers arising in MMTV-PyMT or K14-HPV16 mice, respectively, were negative for REA binding (Fig. 4C and D). The cervical tumors of K14-HPV16/E2 mice were slightly positive for REA peptide binding, but markedly less so than the prostate tumors. Immunohistochemical analysis showed that FITC-REA
peptide colocalized with lymphatic vessels both within tumor tissues (Fig. 4D, first row, middle) and tumor periphery (Fig. 4D, first row, right). This was the case with orthotopic prostate tumor xenografts arising from multiple human prostate tumor-derived cell lines (results for PPC1 tumors are shown in Fig. 4D). This peptide homed to a lesser extent to K14-HPV16/E2 cervical tumors (Fig. 4D). Interestingly, REA phage homed less efficiently to s.c. xenografts of PPC1 than to orthotopic xenografts of the same tumor cell line (Supplementary Fig. 52F). The REA phage strongly bound to PPC1 tumor-derived cell suspensions, but did not bind to cultured PPC1 cells (Supplementary Fig. 52F). Thus, REA seems to primarily recognize prostate cancer lymphatics.

We also asked whether the REA peptide recognizes human prostate cancers by using phage overlay of tissue sections. Immunohistochemical staining with antibodies against lymphatic markers Prox-1 and podoplanin revealed abundant lymphatic vessels in human prostate tumors (Fig. 4D, bottom row, red). Overlay of tissue sections from two primary human prostate cancers with REA phage indicated that this phage recognizes the lymphatics of human prostate tumors (Fig. 4D, bottom row, green). The AGR phage did not bind to the human tumor sections (not shown).

LyP-1 and LyP-2 have different specificities. Given the similar amino acid sequences of the LyP-1 and LyP-2 peptides, and the fact that they both bind to tumor lymphatics, we were interested in comparing their specificities. Surprisingly, these peptides recognize different tumors. Although both peptides homed to the K14-HPV16 skin cancer lymphatics (data not shown), LyP-1 phage homed to MDA-MB-435 breast tumors growing s.c. but not to the de novo cervical tumors, whereas the opposite was true of LyP-2 (Fig. 5A). Both phage did not home to the normal cervix or normal breast tissue. To confirm these differences in specificity, we conjugated one peptide as a fluorescein conjugate and the other conjugated to rhodamine and vice versa. Both LyP-2 conjugates homed to cervical tumors, whereas neither LyP-1 conjugate did so. The opposite result was obtained when the same conjugates were tested in MDA-MB-435 tumor-bearing mice (Fig. 5B). These data indicate that different binding sites exist for the two LyP peptides in different types of tumors.

Lymphatic homing peptide conjugates destroy tumor lymphatics. One potential application of peptides that home and bind to the distinctive lymphatic vasculature of tumors is to target delivery of toxic payloads aiming to disrupt the tumor lymphatics, thereby assessing their functional importance and prospects as a therapeutic target. We began this assessment by linking two of our signature-finding peptides to a toxic agent, assessing its effects on the tumor lymphatics. As a toxic agent, we used conjugates with an apoptosis-inducing peptide, KLAKLAK. In a previous study,
this peptide was linked to blood vascular tumor-homing peptides and shown to be selectively cytotoxic to angiogenic endothelial cells and to have demonstrable antitumor activity (26). To determine whether peptides recognizing tumor lymphatics could be used to target those lymphatics, we synthesized the REA and LSD peptides as conjugates with LKLAKLAK, and systemically treated mice bearing PPC1 or C8161 xenografts. Treatment with the REA conjugate had no effect on tumor blood vessel density in the PPC1 tumors, but significantly reduced the number of tumor lymphatics; the uncoupled mixture had no effect on the lymphatics compared with the PBS control (Fig. 6A). The conjugate had no effect on tumor growth (Fig. 6B), indicating (perhaps not surprisingly) that the tumor-associated lymphatics were not essential for primary tumor growth. Examination of lymphatics in normal skin revealed no discernible effect by the REA or LSD conjugates, and no significant differences were observed in the weight of the mice belonging to the various treatment groups, indicating lack of general lymphatic effects or overt toxicity (data not shown). Reduced density of tumor lymphatics was also seen in C8161 melanoma xenografts of mice treated with the LSD conjugate (data not shown).

To study the mechanism of the lymphatic disruption by the REA conjugate, we examined the frequency of apoptosis in lymphatic endothelial cells in PPC1 tumors using caspase-3 as a marker. The tumors of the mice treated with the REA conjugate had a significant increase in lymphatic endothelial cells expressing active caspase-3 compared with tumors of mice treated with PBS, REA, or a mixture of REA and LKLAKLAK (Fig. 6C). These data indicate that the REA conjugate reduced the lymphatic vessel counts by inducing apoptosis in lymphatic endothelial cells.

### Discussion

In this article, we show an extensive heterogeneity of tumor lymphatics. We have identified peptides that recognize the lymphatics of individual tumor types, including transgenic mouse tumors arising de novo in different organs, as well as human tumor xenografts. We also describe a peptide that distinguishes the lymphatics of premalignant prostatic lesions, both from normal lymphatics and from those of fully developed tumors in the same transgenic mouse model of prostate carcinogenesis. The lymphatic

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*TRAMP, MMTV-PyMT, and K14-HPV16 are genetically engineered mouse models of organ-specific carcinogenesis, each of which presents first with angiogenic dysplasia and subsequently carcinoma.

†The specific homing of phage is considered to be strong (>10-fold compared with control), weak (between 5- and 10-fold), or nonspecific (below 5-fold).

‡Phage homing corroborated by fluorescent peptide homing.
markers detected by the homing peptides are specific for lymphatic vessels (i.e., the peptides do not bind to the blood vessels of the same tumors or premalignant lesions). Several prostate cancers shared the same lymphatic marker. These results show that the lymphatics express a zip code system that is akin to the one in blood vessels, but distinct from it. In beginning to assess the applications of this knowledge, we showed that systemic treatment of tumor-bearing mice with a lymphatic homing peptide linked to a proapoptotic compound could selectively destroy tumor lymphatics.

We used a new screening method based on the immunoselection of lymphatic endothelial cells from whole tumor cell suspensions that had been preincubated with phage display libraries, thereby enriching for phage bound to this rare cell type. This method allowed us to focus on the selection of phage-displayed peptides that identify specific features in the lymphatics linked to proapoptotic compounds.
of the target tissue. We show in each of the tumor models that peptides strongly represented in the selected phage pools specifically homed to tumors, and extensively colocalized with markers of lymphatic endothelial cells in the tumor tissue after an i.v. injection. In contrast, there was no colocalization with blood vessel endothelial markers. Costaining with the lymphatic markers LYVE-1, podoplanin, Prox-1, and VEGFR3 were consistent in supporting this result. The use of multiple markers is an important standard, as none of the lymphatic endothelial markers is completely specific for lymphatics (30, 31). In aggregate, however, they provide strong evidence for a lymphatic vessel identity of the structures that our peptide recognizes in tumors.

We have now shown lymphatic vessel specialization in every one of the five tumor types studied. These tumors consisted of xenograft models of melanoma, breast, and prostate carcinomas, as well as transgenic mouse models developing prostate, skin, and cervical cancers. In addition, the LSD, REA, and LyP peptides each recognized tumor lymphatics in more than one inbred mouse strain. For example, the REA peptide homed to prostate cancers in TRAMP mice (C57BL/6 background) and prostate cancer xenograft tumors grown in nude mice (BALB/c), demonstrating that their specificity is not limited to any given mouse strain. Previous studies have defined a peptide that distinguishes the lymphatics of MDA-MB-435 breast cancer xenografts from normal lymphatics (13, 25). The present results show that such molecular specialization of tumor lymphatics is not limited to this tumor, but is likely to be a generalized phenomenon. The peptides we identified in this study as being specific for lymphatic vessels in the various tumor models were essentially specific for the tumor type used in the screening. Interestingly, the only other tumors with lymphatics recognized by the TRAMP tumor-homing peptide REA were xenograft tumors generated with four different human prostate cancer cell lines. This result suggests that the changes detected by our peptides in tumor lymphatics may be tumor type specific. Similar experiments with tumor blood vessels have revealed two classes of peptides identifying signatures of the angiogenic neovasculature. One pan-specific class recognizes markers that are generally associated with angiogenesis in most tumor types and organs (1, 4, 32, 33), whereas a second class of peptides detected tumor type–specific vascular signatures (7, 8). Although we did not isolate any peptides that identified pan-specific markers of tumor (but not normal) lymphatics, we anticipate that additional screens could reveal such entities.

**Stage-specific lymphatic signatures during tumorigenesis.** We obtained two stage-specific lymphatic homing peptides using different neoplastic lesions in the TRAMP model. The REA peptide selectively recognizes the lymphatics in fully developed TRAMP tumors, whereas the AGR peptide was only reactive with the lymphatics in PIN lesions. Our laboratories have previously obtained homing peptides that distinguish the blood vessels of premalignant lesions from normal blood vessels and those of malignant tumors arising subsequently in the same transgenic mouse models (7, 8). The present results suggest that lymphatic vessels display a similar evolution of molecular specificities as tumorigenesis progresses.

![Figure 5. Differential tumor-homing specificity of LyP-1 and LyP-2 peptides. A, LyP-1 and LyP-2 phage were i.v. injected into mice bearing MDA-MB-435 breast cancer xenografts or K14-HPV16/E7 tumors (n = 3). B, fluorescein-labeled LyP-1 and rhodamine-labeled LyP-2 (top row) were i.v. injected (100 μg of each peptide) into mice bearing tumors or premalignant lesions. Alternatively, the injection consisted of rhodamine-labeled LyP-1 and fluorescein-labeled LyP-2 (bottom row). Tissues were collected and processed for histologic analysis 2 hours later. LyP-1 homes to the MDA-MB-435 tumors, whereas LyP-2 homes to the cervical cancers and premalignant lesions. Phage homing to the premalignant lesions was significantly higher than to the corresponding tumors in both models (P < 0.01). Original magnification, ×400.](image)
A prospective family of lymphatic signatures? The mutually exclusive tumor specificity of the LyP-1 and LyP-2 peptides is interesting given the close sequence similarity of these peptides (CGNKTRGC versus CNRRTKAGC). LyP-1 recognizes lymphatics and tumor cells in MDA-MB-435 and MMTV-PyMT breast cancers (13). Careful comparison of the ability of the two peptides to accumulate in MDA-MB-435 tumors and cervical carcinomas after i.v. injection showed that MDA-MB-435 tumors were positive for LyP-1, whereas cervical carcinomas were not, whereas LyP-2 had the opposite specificity. Interestingly, both peptides bound to other cell types in the neoplastic lesions: LyP-1 binds to and is internalized by breast tumor cells, whereas LyP-2 binds to scattered cells in the neoplastic cervix. The bases for and the implications of these distinctive lymphatic and non-lymphatic binding specificities are presently unclear and deserve future investigation. We have encountered a third peptide in the LyP series, CNKRTRGC. We did not include that peptide in this study but its specificity seems to parallel that of LyP-1 (25). Comparison of the three sequences indicates that shifting the glycine residue from the NH2-terminal to the COOH-terminal end is not important to the specificity of the peptide, but that the arrangement of the basic residues in the K/RTRR/K motif can alter specificity. Our attempts to identify the binding molecules (receptors) for the LyP peptides (and indeed for other of the lymphatic signature–finding peptides identified in this study) have not been successful thus far, and this remains an agenda for future studies. The closely related sequences of the LyP peptides predict the existence of a family of related receptors with tumor type–specific expression in lymphatics. The distinctive specificity of peptides containing the RGD motif in different sequence contexts for individual integrins (34) exemplifies the archetype.

Prospects for therapeutic and diagnostic targeting of the lymphatics. The peptides we describe here have potentially important uses. Early targeting of the tumor lymphatics for destruction may serve to reduce metastatic spread, as lymphatic vessels provide one of the main routes for the spreading of many types of cancer (15–17, 35, 36). In the present study, we were able to reduce the abundance of lymphatics in melanoma xenograft tumors by using lymphatic homing peptides to direct a toxic peptide to the lymphatics in these tumors. In agreement with earlier studies (36), the destruction of the lymphatics had little effect on the growth of the melanoma tumors. These data support the proposition that primary tumor growth is not in general dependent on the lymphatic neovascularure. However, targeted destruction of tumor lymphatics with homing peptide conjugates has prospect to limit metastatic dissemination as has been exemplified in other studies that genetically manipulated lymphatic growth factors to eliminate tumor lymphatics (15–17, 35, 36).

Although studying the effect of the lymphatic homing peptide-drug conjugates on metastasis is one of our long-term aims, we foresee considerable potential in more general applications aimed at producing antitumor effects with homing peptides for tumor lymphatics. Lymphatic homing peptides can be harnessed to deliver a payload into the tumor, as illustrated herein for fluorescein and for the D(KLAKLAK)2 proapoptotic peptide. Targeted drug conjugates can potentially have broader effects than what can be obtained by destroying peritumoral lymphatics. Homing peptides for tumor blood vessels have been used in targeted delivery of therapeutic agents into tumors. As a result of such targeting, the efficacy of the drug increased, whereas its side effects were reduced (26, 32, 37, 38). Our lymphatic homing peptides present another potential route for targeted delivery of drugs into tumors.
Tumor-specific changes in the lymphatics may also have applications in diagnostic molecular imaging of tumor growth, progression, and response to therapy, as well as for early detection of incipient organ-specific cancers (or premalignant progenitor lesions) that evidently have both blood and lymphatic vascular signatures.

Acknowledgments

Received 11/7/2005; revised 2/8/2006; accepted 4/1/2006.

References


Grant support: NIH grants PO1 CA 82713 (E. Ruoslahti and D. Hanahan), PO1 CA 104898, and RO1 CA115410 (E. Ruoslahti); Cancer Center support grant P20 CA 30199 (The Burnham Institute) and other grants from the National Cancer Institute/NIH (D. Hanahan); Department of Defense grant DAMD 17-02-1-0315 (E. Ruoslahti); fellowship DAMD17-02-0399 from the Department of Defense (L. Zhang); and National Cancer Institute training grant T32 CA77109-05 (J.A. Hoffman).

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We thank Dr. Rania Kairouz for her participation in the early stages of this work; Dr. Fernando Ferrer for peptide syntheses; Dr. Daniel Mercola for human prostate cancer samples; Robbins Newlin, Cherry Congenico, and Katie Gilliland for technical assistance with histology; and Roslin Varghese for editing.
Lymphatic Zip Codes in Premalignant Lesions and Tumors

In the article on lymphatic zip codes in premalignant lesions and tumors in the June 1, 2006 issue of Cancer Research (1), the authors' corrections to the text were not included due to a journal error. A pdf of the corrected text is available on the journal website: cancerres.aacrjournals.org. The journal sincerely apologizes for any inconvenience this error may have caused.

Lymphatic Zip Codes in Premalignant Lesions and Tumors

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