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The main problems with current cancer therapies, including those for breast cancer, are that they are only partially effective and highly toxic. The homing peptide technology provides a new targeting strategy that aims at physically concentrating therapeutic agents in tumor tissue by making use of the unique features of tumor vasculature thereby enhancing anti-tumor activity and decreasing side effects. A pentapeptide CREKA, which homes to vasculature of breast tumor in mice, was identified with breast tumor-bearing MMTVPyMT mice by using *in vivo* screening of phage displayed peptide libraries. The CREKA-displaying phage selectively homes to breast tumors in mice relative to other tumors. The selectivity of the phage homing to breast tumors is about 100-fold relative to non-recombinant phage. Fluorescein-labeled CREKA peptide accumulates in breast tumors, but not normal tissues. Mutating the cysteine (C) residue in CREKA to an alanine preserves the homing activity. Receptor identification experiments indicate that the receptor for the CREKA peptide is related to type IV collagen. Additional screenings of phage peptide libraries against extracellular matrix of breast tumor have been performed, and a new method for the identification of homing peptide receptors has been developed. An investigation of drug-CREKA conjugates for in the treatment of breast tumors in mice has been initiated.

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INTRODUCTION

Toxic side effects limit the usefulness of many of the existing anti-cancer drugs. If it were possible to selectively target the drug into the tumor tissue, the efficacy of anti-tumor therapies could be enhanced while simultaneously decreasing the side effects. The homing peptide technology provides a new targeting strategy that aims at physically concentrating therapeutic agents in tumor tissue by making use of the unique features of tumor vasculature (Ruoslahti, E., *Drug Discovery Today* 7: 1138-1142, 2002). In vivo screening of phage displayed peptides library has proved to yield peptides homing specifically to the vasculature of tumor or any given organ (Ruoslahti, E., *Nat. Rev. Cancer* 2: 83-90, 2002). The homing peptides can be used in targeted delivery of therapeutic agents as drug-peptide conjugates. In addition, identification of receptors for homing peptides will provide information for developing the improved versions of homing peptides and their drug conjugates.

In this project, peptides that specifically home to the vasculature of breast carcinoma in MMTV PyMT mice were isolated by in vivo screening of phage displayed peptide libraries. The work has focused an identification of putative receptors for peptides and characterization of peptide/receptor biochemical interactions. The peptide identified in this work may be useful for designing new therapies that specifically target breast cancer.

BODY

The approved tasks for this project are:

Task 1: Identification of the receptors for the peptides specifically binding to the mouse breast vasculature.

Task 2: Characterization of the biochemical interaction of peptide ligands and the putative receptors.

Task 3: Prevention- and intervention-studies with doxorubicin/targeting peptide conjugates in MMTV PyMT transgenic mice.

The progress with each of the Tasks has been made with the past year of grant support of this fellowship:

Tasks 1. We have performed *in vivo* screening of phage displayed peptide library (CX7C) with MMTV-PyMT breast cancer mice by using in vivo phage display technology which developed in our laboratory (Pasqualini and Ruoslahti., 1996; Hoffman., 2004) to identify peptides that selectively home to the vasculature of breast cancers. A phage library (10^9 pfu) was intravenously injected into tumor mice and the bound phage subsequently recovered from breast tumor tissue. The enrichment of phage pool in tumor tissue increased about 100-fold after three rounds of selection; further rounds did not improve the selectivity. Sequence analysis revealed several peptides that had become greatly enriched in the screening. Phage displaying the peptide CREKA (cysteine-arginine-glutamic acid-lysine-alanine) was greatly enriched in the phage pool from the 3rd

round of selection, accounting for about 20% of phage present. As this phage was selected from a CX_7C library, a mutation creating a stop codon in the insert had apparently truncated the peptide into a pentapeptide. *In vivo* homing test of the isolated phage clone showed that the CREKA-displaying phage homed to breast cancer tissue about 130 times more efficiently than non-recombinant T7 phage. CREKA phage did not home to other tissues, including the pancreas, brain, kidneys, and the heart in MMTV PyMT mice. The breast tumor homing of the CREKA phage was specific by the criterion of ligand inhibition, because co-injecting synthetic cognate peptide (5 mg in 500 µl of PBS) with the phage inhibited phage recovery from breast cancer tissue. A peptide that homes to the vasculature of normal breast tissue (CPGPEGAGC; Essler and Ruoslahti, 2002) did not inhibit the homing of the CREKA phage. Similarly, the CREKA peptide did not inhibit the homing of the CPGPEGAGC-phage to normal breast tissue. The CREKA peptide, the best homing peptide from the tumor screening, was chosen for the further study.

As shown in the previous report, abundant fluorescein-labeled CREKA peptide and phage were found in MMTV PyMT breast tumor tissue by immunohistochemistry after an intravenous injection. Whole organ imaging showed striking accumulation of CREKA fluorescence in MDA-MB-435 breast cancer xenografts (Figure 1. FITC-CREKA) did not accumulate in control organs such as the heart.

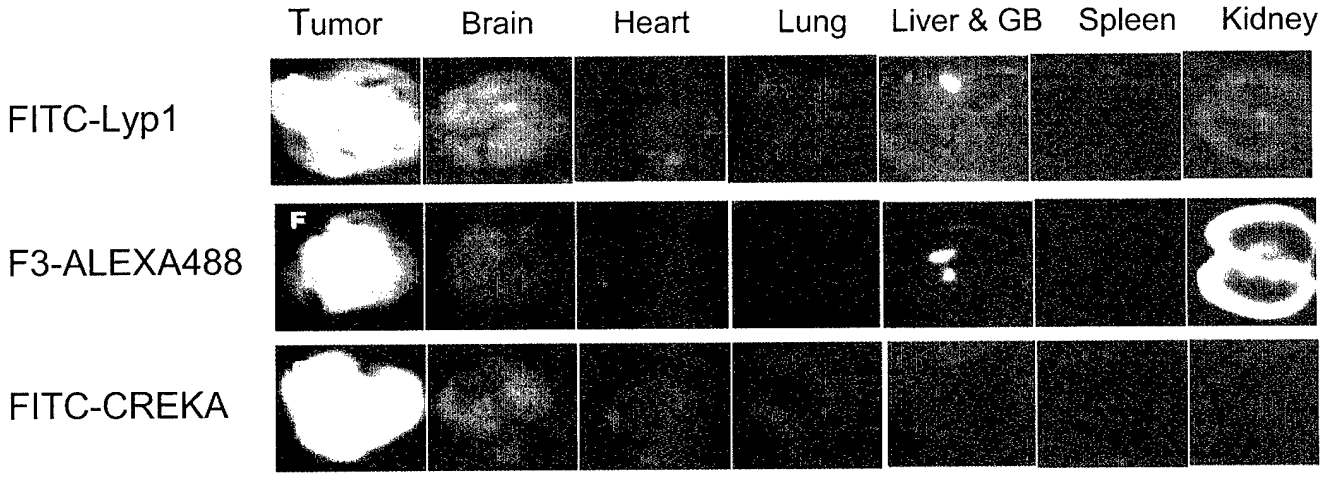

Figure 1. Fluorescein-labeled CREKA peptide homes to tumors, but not to normal tissues. Fluorescein-CREKA peptide (bottom row) and two previously described homing peptides, LyP-1 (Laakkonen et al., 2002) and F3 (Porkka et al., 2002) were intravenously injected into mice bearing orthotopic MDA-MB-435 xenografts. The mice were sacrificed 20 hours later, and the tumors and organs were collected and examined under blue light. Each peptide accumulates in tumor tissue, but not in the normal organs (the gall bladder [GB] is autofluorescent, and the kidneys still contain F3, which at 31 amino acid size may be eliminated more slowly than the smaller LyP-1 and CREKA peptides. There was no detectable fluorescence in tumors of mice injected with a fluorescein-labeled control peptide. (Data produced by AntiCancer Research Inc. in collaboration with us).

To further characterize of the CREKA peptide, we compared the homing specificity of this peptide in different types of tumor models (Figure 2). CREKA-phage (10^9 pfu) was intravenously injected into tumor-bearing mice and phage were recovered from the tissues. The data showed that CREKA-phage, like the peptide, strongly homes to MDA-MB-435 human breast cancer cells xenografts. The specificity of the phage homing was 12-fold over non-recombinant phage. There was some homing to prostate tumors in TRAMP mice or to Krib human osteosarcoma xenografts, but C8161 human melanoma cell xenografts were negative. These data suggest that CREKA selectively recognizes breast cancers *in vivo*.

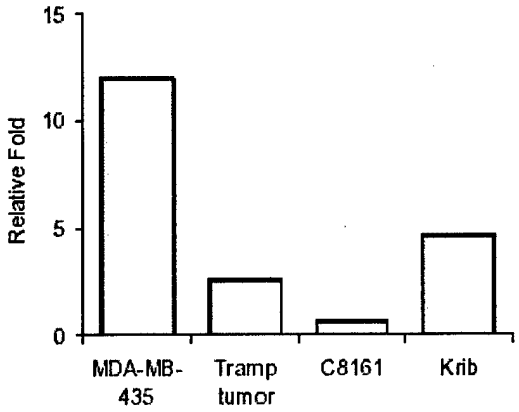

Figure 2. Tumor type homing specificity of CREKA peptide. 10^9 pfu of CREKA phage were intravenously injected into tail vein of tumor bearing mice. After 10 minutes circulation, tumor and control organs were dissected and the bound phage were recovered. Phage were titrated. The number of phage recovered from each tumor type is given relative to the number of phage recovers from tumors of mice injected with non-recombinant phage.

As described in the previous reports, we have obtained evidence indicating that the CREKA receptor is related to type IV collagen (a type IV collagen fragment expressed on phage bound to immobilized CREKA peptide; unpublished). Others have shown that the mRNAs for several collagen chains, including type IV, are greatly (more than 30-fold) elevated in tumor endothelial cells compared to endothelial cells from adjacent normal tissue (St. Croix et al., 2000), various collagens are over-expressed in tumor vessels (Carson-Walter et al., 2001; Netti et al., 2000). Such quantitative differences could explain the selective homing of CREKA-phage and labeled peptide to tumors. Moreover, antibodies thought to recognize cryptic type IV collagen epitopes in tumors have been described (Xu et al., 2001), suggesting that tumor collagens may be poorly assembled in tumor tissue and exposing binding sites for the peptide. However, the binding of CREKA to type IV collagen needs confirmation, as we have not been able to show significant interaction between the CREKA phage and purified type IV collagen. We have developed new technology for the identification and validation of homing peptide receptors (see below).

The CREKA results suggested that the extracellular matrix in tumor vessels and elsewhere in tumors may be modified and could offer a target for homing peptides. To examine this possibility, we devised screening procedure for peptide that recognize extracellular matrix in tumors. The strategies consists of *in vitro* biopanning on matrigel, which is extracellular matrix material isolated from an endodermal mouse tumor, followed by *in vivo* selections in MDA-MB-435 tumor mice. Three rounds *in vitro* biopanning of a CX7C phage library resulted in a 600-fold enrichment of phage pool over non-recombinant phage (Figure 3A). This pool was intravenously injected into tumor-bearing mice, and the bound phage were rescued from the tumor tissue. Thirty-six individual phage clones from the phage pool of 3rd round *in vivo* selection (Figure 3B) were analyzed by sequencing. We also performed the *in vivo* selection and *in vitro* biopanning in a reverse order. Three rounds *in vivo* selection of phage library yielded an 18-fold enrichment of a phage pool (Figure 3C), which when was used additional *in vitro* selection on matrigel gave a 500-fold enrichment (Figure 3D). The peptide-encoding inserts of 36 individual clones from the latter selection were sequenced. Interestingly, a peptide, CSGRRSSKC, appeared multiple times among the sequenced phage clones from both screening strategies. Thus, CSGRRSSKC could be a candidate

peptide that targets extracellular matrix of breast tumor. Characterization of this peptide and the other phage pool (Fig. 3) is underway.

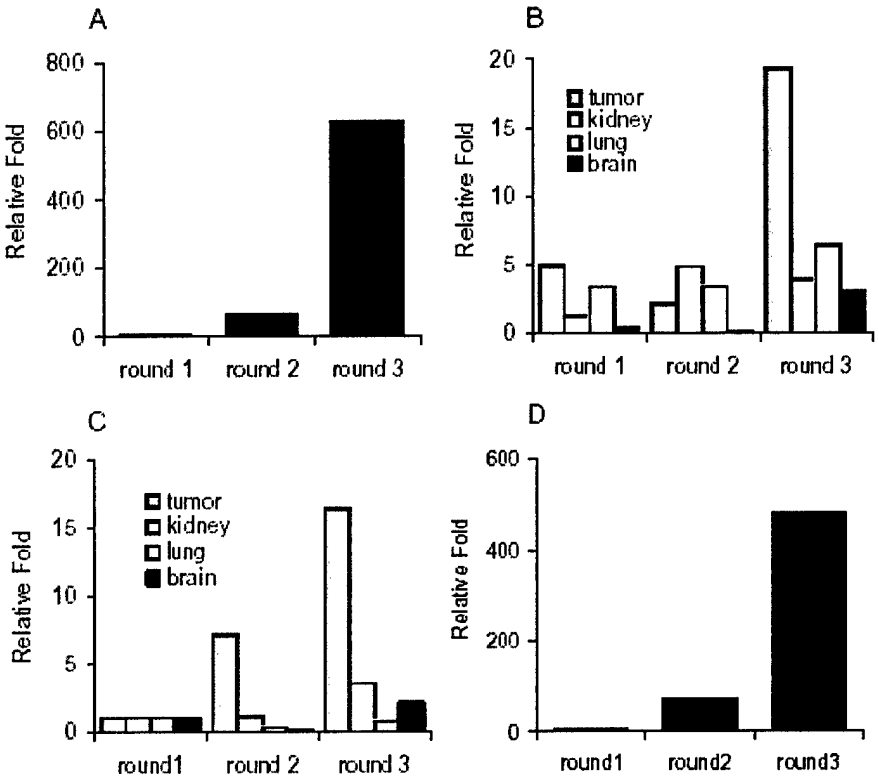

Figure 3. Screening of peptides targeting extracellular matrix. A. *In vitro* biopanning of CX7C library. 96-well plates were coated with 100 ul of matrigel (0.1mg/ml), and then incubated with 1.5×10^{10} pfu phage overnight at 4°C. The bound phage were recovered and amplified for the next cycle of biopanning. A phage pool with 600-fold enrichment was obtained after three rounds of biopanning. B. *In vivo* selection in MDA-MB-435 tumor mice. 3×10^{10} phage from the 3rd *in vitro* biopanning were intravenously injected into tumor mice. The phage bound to tumor tissue were rescued and amplified. After three rounds of selection, a phage pool with an 18-fold enrichment was obtained. There was only non-significant enrichment in control organs. C. *In vivo* selection of CX7C phage library for homing to MDA-MB-435 tumors. After three rounds of selection, a phage pool with 16-fold enrichment in tumor was obtained. D. This pool was further selected by *in vitro* biopanning on matrigel. After three round of *in vitro* biopanning, a phage pool with 600-fold enrichment was obtained.

Task 2. To improve our ability to identify homing peptide receptors, we have recently developed a new method that allows us to simultaneously isolate homing peptides and their receptors. The method was developed as a proof of principle experiment by using normal heart as the target tissue because it is easier to work with normal mice than tumor mice. The first experiments worked so well that we decided to publish the results separately (Zhang et al., submitted). The method makes use of bacterial 2-hybrid screening (Fig. 4). Applying it to the present project will greatly accelerate the progress of identifying receptors for homing peptides. The procedure is performed on phage pools, but can also be used for individual peptides. When performed on pools, it selects for peptides for which a receptor can be isolated from cDNA libraries.

The method is as follows: Phage libraries are screened by the *in vivo* or *ex vivo/ in vivo* procedures described in the application. Once a pool of phage that homes to the target tissue is obtained, the peptide-encoding DNA inserts from the phage pool are amplified by PCR, and the PCR products are subcloned into the bait plasmid pBT of a bacterial two-hybrid system

(BacterioMatch®; Stratagene). This system detects protein-protein interactions based on transcriptional activation of the Ampicillin resistance reporter gene, and a second reporter gene β-galactosidase. Interaction between a peptide present in the selected phage pool and its receptor (encoded by a cDNA from the target tissue) is indicated by growth on selective plates. The nucleotide sequences encoding the peptide and the receptor (usually a fragment of the receptor) can then be determined. Five potential peptide-receptor pairs were obtained. All of the peptides selectively home to the heart vessels; the most potent peptide among them shows over 300-fold accumulation in the heart vessels compared to controls. Four of the receptors have been confirmed by antibody staining and/or *in situ* hybridization as being highly expressed in heart vasculature (the fifth receptor clone is an EST that we chose not to work with). The same method will be applied to the present project in the analysis of receptors for novel breast cancer homing peptides as well as for known peptides such as CREKA and LyP-1.

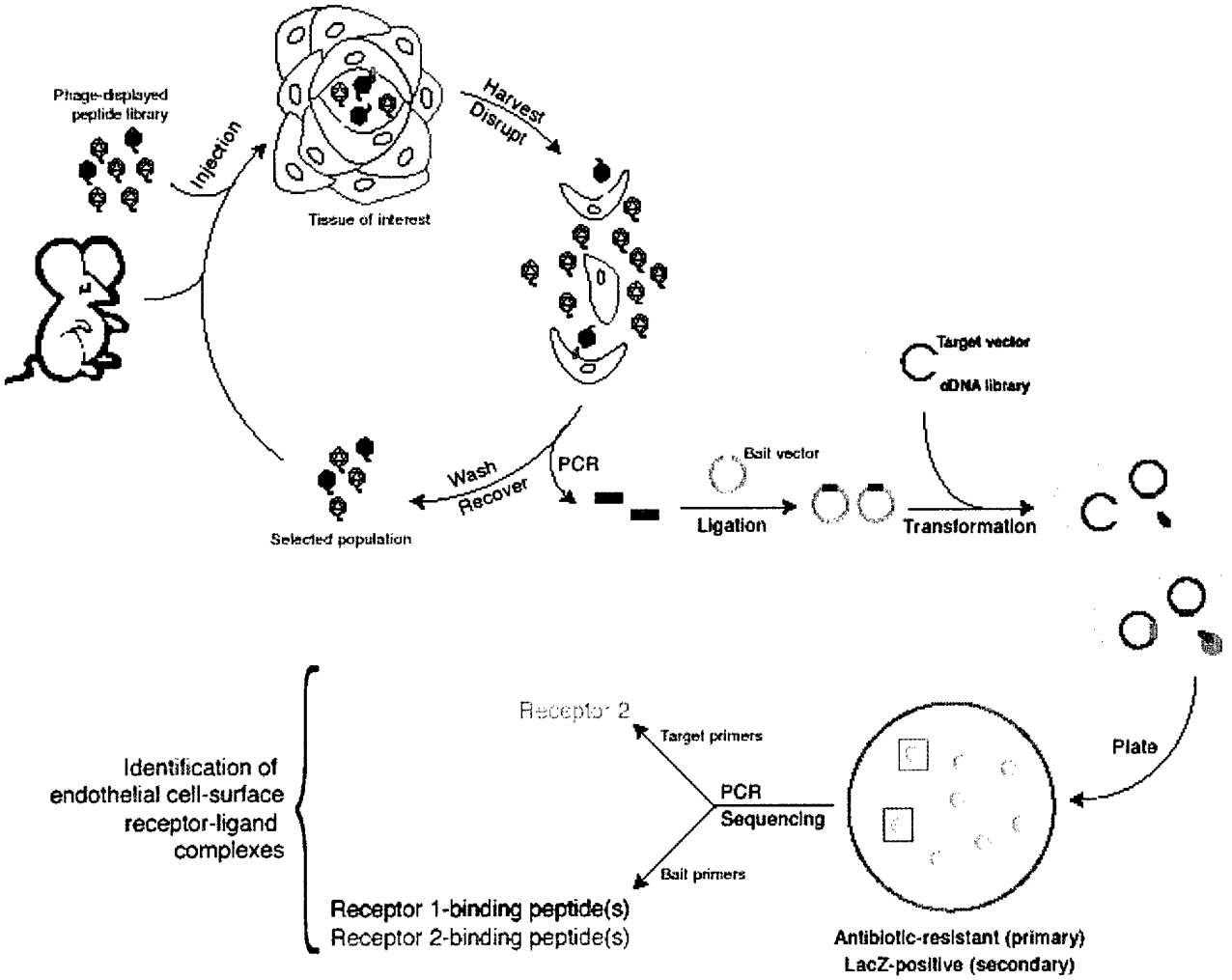

Figure 4. Identification of homing peptide/receptor pairs by *ex vivo/in vivo* phage display and bacterial two-hybrid analysis. The principle of parallel homing peptide isolation and receptor identification. A phage-displayed peptide library is injected into a mouse and allowed to circulate; tissues are harvested and then disrupted into single cells with bound phage. Non-bound phage are washed away, and bound phage are recovered, grown up in bacteria, and the process is either repeated or the peptide-encoding inserts are amplified by PCR and shuttled into a bait vector of the bacterial two-hybridization system. Once in the bacteria, the poolenriched for endothelia-binding peptides can interact with possible receptors, and bestow carbenicillin-resistance to bacteria. Resistant clones are plated on X-gal-containing agar for a secondary screen; target and bait

inserts are amplified and sequenced from carbenicillin-resistant, LacZ-expressing cells (**A**). By the third round, a selected pool bound *ex vivo* heart cells about 230-fold more than non-recombinant T7 phage (**B**). When further selected for homing to the heart *in vivo*, this pool accumulated in the heart vasculature about 190 times more than non-recombinant control phage (**C**). Peptide-encoding inserts from this final pool were ligated into the bait vector, and some bacteria transformed with both the peptide-bait vectors and heart cDNA library (in target vector) were able to grow on 500 μg/ml carbenicillin plates (**D**). Some clones were also positive for the secondary marker, LacZ, as evidenced by the production of the blue color on plates containing X-gal (**E**). (L. Zhang et al, submitted).

Task 3. In preparation for treatment experiments, an independent study was carried out at Anticancer Inc. in San Diego, California compared the tumor homing of fluorescent-labeled CREKA and two particularly effective peptides Lyp-1 and F3 from our laboratory (Fig. 1). CREKA was found to be the most efficient and selective in homing to MDA-MB-435 breast cancer xenografts. These data suggests that CREKA can be used in targeted delivery of therapeutic agents as drug-peptide conjugates for breast cancer therapeutics.

The sulfhydryl group of the cysteine residue in CREKA is potentially the most suitable group for drug conjugation. In preparation for the coupling of the CREKA peptide to drugs, we tested the role of the cysteine side chain in the tumor homing. We synthesized a variant, AREKA, in which the cysteine residue is converted to an alanine. This change had no noticeable effect on the ability of the the fluorescein-conjugated peptide to accumulate in MMTV-PyMT tumors after an intravenous injection (Figure 5).

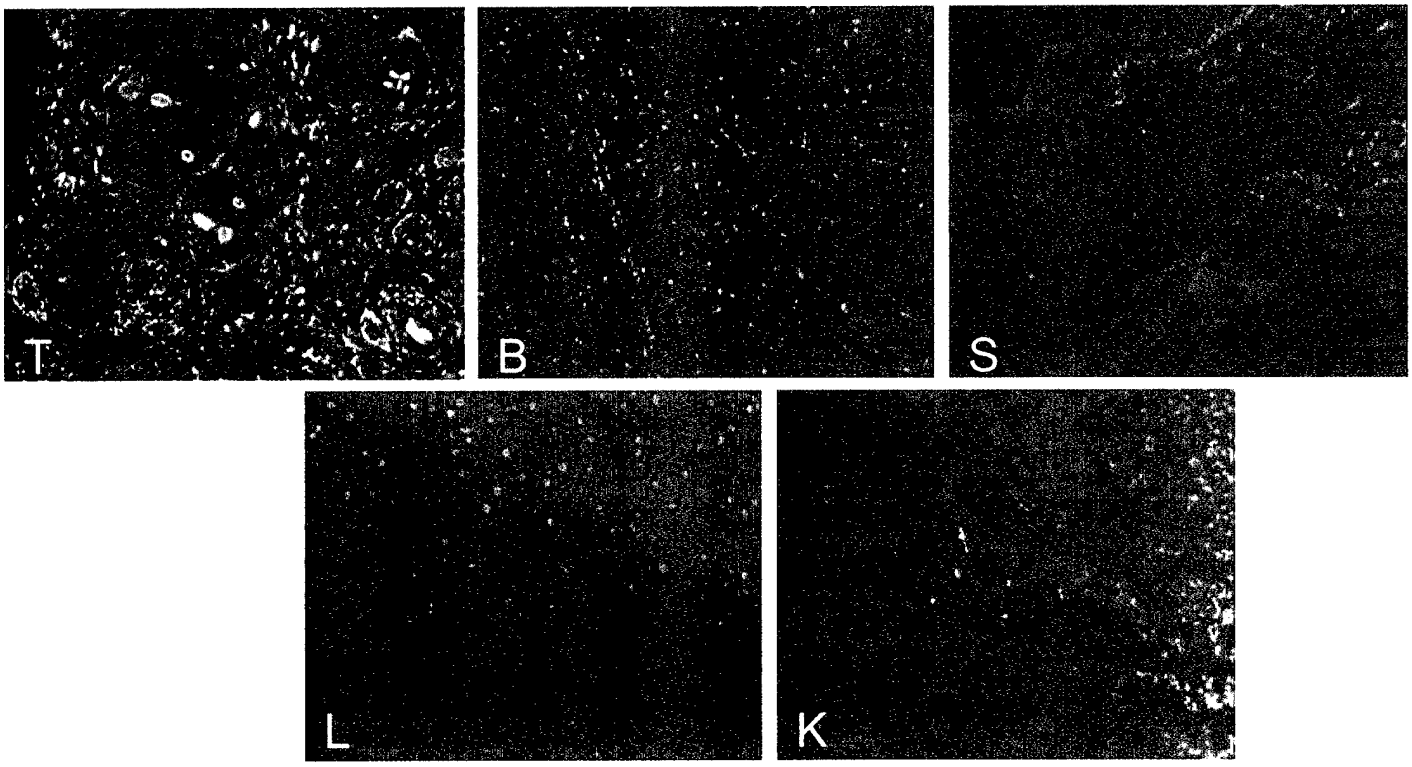

Figure 5. FITC-AREKA peptide homes to breast cancer. 100 μg of FITC-AREKA peptide was injected into the tail vein of tumor-bearing MMTC-PyMT mice. After 30 min of circulation, tumors and control organs were collected, sectioned, fixed, counter-stained with DAPI, and examined in fluorescence microscopy. T, tumor; B, brain; S, skin; L, liver; K, kidney.

We also have collaborated with Dr. Huber (Max Planck Institute for Biochemistry, Germany) in preparing a novel type of peptide-targeted conjugate. Dr. Huber's group incorporates cytotoxic

amino acid to proteins that become toxic when the protein is degraded and the individual amino acids are released. They have prepared CREKA-toxic GFP recombinant proteins for us to test for anti-tumor activity. *In vitro* experiment showed that CREKA-toxic GFP did not significantly affect the viability of MDA-MB-435 cells. We are in the progress of testing the conjugates for *in vivo* anti-tumor activity. The alternative approach such as CREKA conjugated doxorubicin is planned.

KEY RESEARCH ACCOMPLISHMENTS

- Characterized a pentapeptide, CREKA, which specifically homes to breast tumor vasculature
- Identified the type IV collagen as a possible receptor for the CREKA peptide
- Showed that a fluorescein-labeled CREKA peptide accumulates in breast cancer xenografts with a superior efficacy after an intravenous injection.
- Identified a new peptide, CSGRRSSKC, as a candidate peptide that targets extracellular matrix of breast cancers.
- Shown that that the side chain of the cysteine residue in the CREKA peptide is dispensable, for homing peptide activity, providing an excellent site for drug coupling.

REPORTABLE OUTCOMES

A manuscript describing the new identification technology for homing peptide receptors has been submitted.

CONCLUSIONS

The significant progress has been made toward completing each of the Tasks in the application. A new 5-amino acid homing peptide, CREKA, has been shown to be remarkably efficient and specific in its accumulation in breast cancer xenografts. This peptide appears to target the extracellular matrix of breast cancers. Additional peptides with similar characteristics have been isolated. A novel technology has been developed for the identification homing peptide receptors. Finally, progress has been made with the drug conjugation strategies for the CREKA peptide.

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