Models and Technologies

Novel Target for Peptide-Based Imaging and Treatment of Brain Tumors

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Abstract

Malignant gliomas are associated with high mortality due to infiltrative growth, recurrence, and malignant progression. Even with the most efficient therapy combinations, median survival of the glioblastoma multiforme (grade 4) patients is less than 15 months. Therefore, new treatment approaches are urgently needed. We describe here identification of a novel homing peptide that recognizes tumor vessels and invasive tumor satellites in glioblastomas. We demonstrate successful brain tumor imaging using radiolabeled peptide in whole-body SPECT/CT imaging. Peptide-targeted delivery of chemotherapeutics prolonged the lifespan of mice bearing invasive brain tumors and significantly reduced the number of tumor satellites compared with the free drug. Moreover, we identified mammary-derived growth inhibitor (MDGI/H-FABP/FABP3) as the interacting partner for our peptide on brain tumor tissue. MDGI was expressed in human brain tumor specimens in a grade-dependent manner and its expression positively correlated with the histologic grade of the tumor, suggesting MDGI as a novel marker for malignant gliomas.

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Introduction

Glioblastoma multiforme, the most aggressive and most common primary brain tumor in adults, comprises approximately 60% to 70% of all gliomas. Tumors of glial cell origin are graded on a World Health Organization scale as grade 1 to 4. Only grade 1 tumors are considered benign; they grow slowly with a clear tumor–brain interphase enabling the surgical removal of the tumor. Importantly, nearly all low-grade tumors eventually progress to high-grade malignancies (1). Owing to their infiltrative growth, gliomas are associated with high morbidity and mortality (2). Even with the most efficient therapy combinations, median survival of glioblastoma multiforme patients (grade 4) is less than 15 months and most patients die within 2 years with 5-year survival less than 3% (3, 4). In addition, malignant gliomas almost always recur. The recurrent gliomas are very aggressive with one-year survival rate less than 25%.

Therefore, new therapeutic approaches are urgently needed. One of the main challenges is to identify means by which block invasion and recurrence by destroying the invasive tumor satellites that have left the main tumor mass and that cannot be surgically removed. One attractive approach would be to develop drugs/inhibitors that specifically target these satellite tumor cells and the blood vessels they co-opt for growth.

The distinct molecular signatures in the normal and neoplastic tissues have been efficiently exploited using the in vivo phage display screening to identify tissue-specific markers and to survey disease-specific differences (5). Using this technology, several peptides that specifically target the blood vessels in various normal tissues and in tumors (6–8) have been isolated. Tumor lymphatic vessels have also been targeted in this manner (9–11). Such peptides have been used for targeted delivery of drugs, oligonucleotides, imaging agents, liposomes, nanoparticles, and viruses (12, 13).

To identify peptides selectively targeting invasive satellites of malignant gliomas, we performed an in vivo phage display screen using a tumor model that reproduces the infiltrating glioblastoma phenotype (14, 15). Because these tumor cells lack HIF-1, they are incapable of inducing tumor angiogenesis and rely on co-opted normal vessels for growth. We describe a peptide named “CoolP” that specifically homes to invasive tumor satellites and the vessels in these tumors and other glioblastomas.
Materials and Methods

Cells

Hif-1α-deficient (HIFko) and VEGF-overexpressing mouse astrocytes were propagated as described (15). U87MG human and BT4C rat glioma cells (gift from Seppo Yiä-Herttuala, University of Eastern Finland, Kuopio, Finland) were maintained in Dulbecco’s modified Eagle medium (DMEM), 10% fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. MDA-MB-231 cells (gift from Jorma Keskiöja, University of Helsinki, Helsinki, Finland) were cultured in RPMI-1640, 10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and U2-OS (gift from Mariikki Laiho, University of Helsinki, Helsinki, Finland) cells used for lentivirus production were maintained in high-glucose (4.5 g/L) DMEM. Cell lines were obtained during years 2006–2009. No authentication has been done by the authors.

Generation of stable cell lines

To establish U87MG cells stably expressing MDGI-GFP fusion protein, MDGI gene was cloned to a lentiviral expression vector (pBOB\cag\GFP) containing GFP (gift from Seppo Yiä-Herttuala, University of Eastern Finland, Kuopio, Finland). Lipofectamine 2000 (Invitrogen) was used to complex the GFP/MDGI or GFP–containing expression vector and lentiviral packaging plasmids pLP1, pLP2 and pLP-VSVG (Invitrogen) according to manufacturer’s instructions. Virus-containing supernatants of transfected 293FT cells were collected at 72 hours and concentrated at 50,000 × g. Purity of the peptides was determined by an analytical HPLC.

In vivo phage display

We used an NNK-encoded Cx-C peptide library (gift from Erkki Ruoslahti, Sanford-Burnham Institute, La Jolla, CA) on the T7Select415-1-phage (Novagen). Phage selections were performed as previously described (16). For the first ex vivo round, tumor-derived cell suspension was incubated overnight at 4°C with 5 × 10⁷ plaque-forming units (PFU) of the library. Bound phage was rescued and amplified in E. coli (BLT5615, Novagen) and used for the second round of ex vivo selection. Ex vivo enriched phage pool was injected into the tail vein of intracranial HIFko tumor-bearing mice, and allowed to circulate for 15 minutes. Brain, including tumor, was excised, and the recovered and amplified phage was used for the next rounds of in vivo panning. In each round, nonrecombinant control phage was injected to a separate mouse to assess the background.

Peptide synthesis

CooP (NH2-ACGLSLGVLGA-CONH2) and its control peptide (NH2-ACVAALNADG-CONH2) were synthesized using an Apex 396 DC multiple peptide synthesizer (Advanced ChemTech) with Fmoc strategy and O-Benzotriazole-N,N,N,N'-tetramethyl-uronium-hexafluorophosphate (HBTU, GLS Biochem) and N,N-diisopropyl-methylamphotilane (DIPEA, Fluka) as coupling reagents, and rink resin as solid support (Novabiochem). DTPA was conjugated directly to the alpha-amino group of the peptide. For radiolabeling, DTPA-conjugated peptides (20 µg per animal) were mixed with 0.2 mol/L NaAc (pH 5) followed by addition of 5 MBq of 111Indiumchloride(111In, Mallinckrodt) per animal. Reaction mixture was incubated for an hour at room temperature and radiochemical purity was measured using an instant thin-layer chromatography (ITLC-SG, Pall Corporation). Radiochemical purity of the peptides was 99% to 100%.

Isothiocyanate-activated fluorescein (FITC) was conjugated to peptides on resin via an additional N-terminal lysine. After removal of Fmoc group using 20% piperidine in dimethyl formamide (DMF)-free N-terminus was acetylated with 20% acetic anhydride in DMF. Acid labile methyltrityl protection group of the additional lysine was removed with 2% trifluoroacetic acid in DCM. FITC (2 mg) was added into reaction mixture following addition of 15 µL triethanolamine. Conjugated peptides were cleaved from the resin using a mixture of 95% trifluoroacetic acid (TFA), 3% ethanedithiol (EDT), 1% triisopropylsilane, and 1% H2O. Peptides were purified with reverse-phase high-performance liquid chromatography (RP-HPLC) using C18 reverse phase column (xTERRA, 250 × 10 mm, Waters) and an acetonitrile gradient (ACN, 0%–95%, 45 minutes). Purity of the peptides was determined by an analytical HPLC.

CooP-CPP-Cbl (NH2-CGLSLGLGVA-CONH2) and its control peptide were synthesized on an ABI433A peptide synthesizer (Applied Biosystems) by t-Boc chemistry using 4-methylbenzhydrylamine-polystyrene resin as solid support (Novabiochem). DTPA was conjugated directly to the alpha-amino group of the peptide. First amino acid Boc-Lys(Fmoc)OH was coupled manually. All subsequent couplings used HOBt (1-hydroxy-7-azabenzotriazole) and N,N'-dicyclohexyl-carbodiimide to couple standard Boc protected amino acids. Fully protected peptidy resin was removed from synthesizer and treated manually to generate free ε-amino of C-terminal lysine using 20 vol% piperidine in DMF for 20 minutes followed by additional 40 minutes in 20% piperidine in 1 vol% Triton X-100 DMF/DMF/NMP 1:1:1 v/v/v. Chlorambucil (10 equivalent) was added. Coupled 2-[1H-benzotriazol-1-yl]-1,1,3,3-tetramethyluronium tetrafluoroborate, 1-hydroxybenzotriazole and DTPA in DMF/NMP (1:1, v/v) for 1 hour at room temperature. The freeze-dried peptidyl resin was treated with anhydrous hydrogen fluoride containing 10 vol% scavengers (p-cresol and p-thiocresol, 1:1) at 0°C for 1 hour. After evaporation of hydrogen fluoride, residue was washed with diethyl ether and extracted with 10 vol% scavengers (p-cresol and p-thiocresol, 1:1) at 0°C for 1 hour. After evaporation of hydrogen fluoride, residue was washed with diethyl ether and extracted with 10...
vol% acetic acid, and lyophilized. Peptide was purified by RP-HPLC (Discovery C-18 column, Supelco, Sigma-Aldrich) using a linear gradient of ACN/water containing 0.1 vol% TFA (20%–60% ACN, 50 minutes). Identity of the purified product was verified by analytical RP-HPLC and by Perkin Elmer proTOF 2000 matrix-assisted laser desorption ionization time-of-flight mass-spectrometer. Mass spectra were acquired in positive ion reflects mode using α-cyano-4-hydroxycinnamic acid as matrix (10 mg/mL, 3/7 acetonitrile:water, 0.1% TFA).

**SPECT/CT imaging**

For a combined small-animal SPECT/CT (single photon emission computed tomography–computed tomography; Gamma Medica) imaging, radiolabeled peptide (20 μg in 100 μL) was injected into the tail vein of intracranial U87MG tumor-bearing mice. Medium energy parallel hole collimators were used with a 171/245/416 keV energy window for 111In. Planar two-dimensional (2D) imaging was acquired at earliest possible time point after injection with 60 s acquisition time/image up to 30 minutes. For three-dimensional (3D) imaging total of 64 projections, (120 s/projection) were used. Matrix size was worth six-order low-pass post-filtering with 0.3 cut-off frequency to obtain 3D images with dimensions of 512 × 512 matrix size in 87 mm3 FOV. Both CT and SPECT images were interpolated into final 256 × 256 × 256 size by using commercial software (Gamma Medica). Images were combined using IDL software (ITT Visual Information Solutions) and monitored with the Amira software (Mercury).

**Analysis of radioactivity in organs**

After the last imaging session, animals were sacrificed using CO2 and tissue samples were collected, weighted, and radioactivity was measured in an automated gamma counter. Obtained counts were corrected for background radiation and physical decay. Tissue radioactivity was expressed as percentage of the injected dose per gram of tissue (% ID/g ± SD).

**Detection of peptides and antibodies in tissues**

Intravenously injected fluorescein-labeled peptides (100 nmole) or antibodies (20 μg/animal) were allowed to circulate for 1 hour. To detect in vivo distribution of the fluorescein-labeled peptides, frozen sections (5–10 μm) were stained with rabbit anti-FITC antibodies. To visualize blood vessels, MDGI, and tumor cells, sections were stained with rat anti-mouse PECAM-1/CD31 (BD Pharmingen), goat or rabbit anti-MDGI (Santa Cruz Biotechnology) or rabbit anti-SV40 large T antigen [gift from Dr. Hanahan, Swiss Institute for Experimental Cancer Research (ISREC), Lausanne, Switzerland] antibodies followed by Alexa-594 or Alexa-488 conjugated secondary antibodies (Molecular Probes/Invitrogen). Nuclei were visualized with 4’, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Sections were examined under an inverted fluorescence microscope (Zeiss Axioplan).

**Yeast-2-hybrid screen**

Sense- and antisense oligonucleotides (AATCTCGCG-GACTGAGCGGGTTAGCGTTGCTG, GATCCAGCA-ACGCCTAACCCTGCTGAGCAG) encoding the CooP sequence were cloned into a pGBK7 bait vector (Clontech Laboratories). Mouse embryonic (E12.5) library in the Pad-GAL4-2.1 prey vector was transformed into yeast cells expressing the baits. Clones producing β-galactosidase were isolated and the prey cDNAs were sequenced. Screen was performed according to the Fields method (17) at the Yeast-Two-Hybrid Core Facility (University of Helsinki, Helsinki, Finland).

**Cloning of the MDGI from brain tumor cDNA**

RNA was isolated from brains of intracranial HIFko tumor-bearing mice using the Rneasy kit (QIAGEN). RNA from two brains (1 μg) was used for cDNA synthesis by the QuantiTech RT Kit (QIAGEN). Full-length MDGI was cloned to the pcDNA3-9E10 plasmid using the following primers: forward: GGAATTCGGAGCCCTTGTGCG-TACCTGGAAG; reverse: CCTCAGATCACGCCTCTT-CTCATAAAGTCCAGTGCTC.

**Tumor xenografts and treatment with targeted Cbl**

Athymic female Balb/c nu-nu mice (4–6 weeks) were used in tumor experiments. Establishment of intracranial tumors has been described (15). Mice were used for experiments 10 to 21 days after tumor cell inoculation. To establish subcutaneous tumors, 5 × 106 cells were grafted on the abdominal side of the mice. For treatment study, intracranial HIFko tumors were established and treatment was started 5 days after implantation. Mice were randomly divided in groups (4 mice/group) and injected intravenously with PBS, CooP-CPP-Cbl (5 mg/kg), or free Cbl (same molar amount as CooP-CPP-Cbl) every second day. Weight of the animals was monitored during the study. Animal studies were conducted according to guidelines of the Provincial Government of Southern Finland and protocol was approved by the Experimental Animal Committee.

**Murine and human tumor material**

Formalin-fixed paraffin-embedded tumor samples obtained from patients diagnosed with brain tumor were retrieved from archives of the Department of Pathology, Helsinki University Central Hospital, Finland. Study was approved by an Ethics Committee of Helsinki University Central Hospital, Finland. A permission to use tumor tissue for the study was obtained from the Ministry of...
Social Affairs and Health, Finland. In addition, formalin-fixed paraffin-embedded sections of murine xenografts derived from EGFRvIII transformed, INK4/Arf/−/− neural stem cells (NSCG) and from a glioblastoma multiforme patient-derived tumor (GBM43; ref. 18) were used for the immunohistologic studies. Antigen retrieval was performed by heating the samples in 10 mmol/L sodium citrate buffer (pH 6.0) using a microwave oven (780W 5 minutes, 380W 10 minutes). MDGI was detected from the sections using TSA Indirect Kit (PerkinElmer).

To better visualize the localization of MDGI in gliomas, double immunofluorescence staining of human glioma samples using antibodies against MDGI andPECAM-1 (endothelial marker) was performed. After deparaffinization, sections were blocked with blocking solution (5% BSA, 0.1% Triton X-100 in PBS) and incubated with the appropriate primary antibodies at +4°C overnight. After the PBS washes, sections were incubated for 1 hour at room temperature with fluorescein labeled secondary antibodies (Molecular Probes/Invitrogen) and Hoechst (1 μg/mL) in PBS to visualize the nuclei.

Results

Identification of peptides homing to malignant brain tumors

Because of the infiltrative growth, glioblastomas contain invasive tumor satellites that cannot be surgically removed and that contribute to recurrence of the tumors. We performed a combined ex vivo/in vivo phage display screen (16) to isolate peptides capable of homing to these invasive satellites that are nurtured by co-opted vasculature. As a model, we used the hypoxia-inducible factor–deficient transformed murine astrocytes (HIFko) that are incapable of angiogenesis and adapt to that inability by co-opting existing normal vessels, which leads to perivascular invasion and growth as invasive tumor satellites (14, 15).

Two rounds of ex vivo selection using the CX:C phage-displayed peptide library on cell suspensions from intracranial HIFko tumors yielded a 5-fold enrichment of phage binding to the tumor-derived cells compared with the control (Fig. 1A). We subjected this preselected phage pool to in vivo selection to screen for peptides capable of homing to the tumors after intravenous injection. We observed an increase in tumor homing over the control phage from 95-fold in the first in vivo round to approximately 200-fold in the third round (Fig. 1A). After the third in vivo round, we sequenced approximately 60 randomly selected individual clones and searched for enriched peptide sequences. On the basis of the sequence data, we chose five clones to validate their ability to bind and home to the HIFko brain tumors. Phage 11, 34, 36, 50, and 59 all showed high, up to 1,700-fold, ex vivo binding to tumor cell suspensions over the control phage. However, phage 11, 50, and 59 showed even higher affinity for the normal brain (Fig. 1B) suggesting that these phage/peptides were rather brain than brain tumor specific. The brain tumor to normal brain ratio was about 13 for phage 34 and over 80 for phage 36 demonstrating preference for brain tumor tissue (Fig. 1B). Peptides encoded by phage 34 (CSESGLGVA) and 36 (CGLSGLGVA) shared 7 of 9 amino acids. When we tested the in vivo homing of these phage to the intracranial HIFko tumors, phage 36, hereafter referred to as CooP, showed nearly 20-fold homing to the tumor, while only negligible accumulation in other organs, including liver, kidney, lung, and normal brain, was detected (Fig. 1C). Phage 34 showed only modest tumor homing, about 3-fold over the control phage (data not shown).

To confirm that the homing was mediated by the CooP peptide and to study the tissue localization of the peptide more precisely, we conjugated the synthetic peptide to FITC. We injected the FITC-CooP intravenously to mice bearing intracranial HIFko tumors and allowed it to circulate for 60 minutes. The peptide accumulated in the tumor satellites in the brain (Fig. 1D and E). Identification of the tumor satellites was confirmed by staining adjacent tissue sections for SV40 large T antigen to visualize the tumor cells (Fig. 1F). We detected no CooP peptide in the surrounding histologically normal brain (Fig. 1E) or other tissues examined (data not shown). Some peptide-derived fluorescence was observed in the kidneys and the choroid plexus, a cerebrospinal fluid secreting epithelial structure located in brain ventricles. Since a control peptide (E3; ref. 19), and the previously reported tumor-homing peptides F3 (19), and LyP-1 (10) used as additional controls, also produced a similar fluorescent pattern at these sites (data not shown), the signals in these organs were most likely produced by nonspecific peptide or fluorescein uptake related to excretion of the peptides.

To assess tumor-type specificity of the CooP peptide, we tested its homing to other types of brain tumors. Intravenously injected CooP peptide homed to intracranial U87MG human glioma (Fig. 1G) and rat BT4C glioma xenografts (Fig. 1H). No CooP peptide was detected in subcutaneous tumors established from the HIFko cells (Fig. 1J). CooP peptide also did not home to tumors derived from VEGF-expressing HIFko cells (15), which contained a massive network of angiogenic blood vessels (Fig. 1K).

Mammary-derived growth inhibitor is the binding partner for the CooP peptide

To identify protein(s) to which the CooP peptide binds in the target tissue, we performed a yeast-two-hybrid screen (17). The peptide sequence was introduced into the bait plasmid and cotransformed to bacteria with the target plasmids encoding a murine embryonic (E12.5) cDNA library. An embryonic library was chosen because the CooP peptide did not home to the normal brain, and many embryonic proteins become upregulated in tumor tissue but are absent in normal adult tissues (e.g., c-Myc, the EDB domain of oncofetal fibronectin; ref. 20, 21) and the large isoform of tenasin C (22). The yeast-two-hybrid screen yielded ten in-frame hits, which were used for sequence identification from the BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). These cDNAs
represented four different proteins: (i) an eukaryotic translational initiation factor (EIF3S7), (ii) a neural peptidase (CPE), (iii) a mammary-derived growth inhibitor (MDGI), and (iv) an expressed sequence tag (EST). Three of the hits represented two different cDNAs for MDGI. Only MDGI expression increased binding of CooP displaying phage to the transiently transfected U2-OS cells 10-fold compared with the original library (Supplementary Fig. S1A, for methods see Supplementary Information). MDGI expression in transfected cells was confirmed by Western blot analysis (Supplementary Fig. S1B, for methods see Supplementary Information). After this initial validation, MDGI, also known as the fatty-acid binding protein 3 (FABP3) or the heart-type fatty acid-binding protein (H-FABP) was further investigated as a potential interacting partner for the CooP peptide.

Figure 1. Identification of the CooP peptide that homes to a subset of brain tumors. A, phage screen comprising two ex vivo (Ev) rounds performed by incubating phage library with intracranial HIFko tumor-derived cell suspension followed by three in vivo (iv) rounds with intravenously injected phage pool into the mice bearing intracranial HIFko tumors. Phage enrichment is shown as fold increase over the control phage. B, individual phage from the third in vivo selection round were tested for the ex vivo binding to the cell suspension derived from tumor containing brain and normal brain. Graph shows phage binding to the brain tumor relative to the normal brain. C, homing of the CooP phage to brain tumor, histologically normal brain, liver, kidney, and lung tissue compared with the control phage. Values represent a mean of three experiments. Error bars, ± SEM. D–F, fluorescein-conjugated CooP peptide (100 μmol/L, 100 μL/animal) was injected into the tail vein of mice bearing intracranial tumors and allowed to circulate for 60 minutes followed by perfusion of the mice through the right ventricle of the heart and excision of the tumor. Staining of the tumor sections with an anti-fluorescein antibody (red in E) shows accumulation of fluorescein-conjugated CooP in HIFko clusters identified by staining of an adjacent tissue section with an antibody against the SV40 large T antibody (green in F), a marker for tumor cells. Homing of the fluorescein-conjugated CooP peptide (red) to intracranial U87MG (G) and BT4C (H) gliomas. Absence of CooP homing to subcutaneous MDA-MB-231 breast tumors (I), to subcutaneous HIFko tumors (J), and to the VEGF-overexpressing intracranial tumors (K). G–K, sections were stained with an anti-PECAM1 antibody (green) to visualize tumor vasculature. D, F–K, nuclei were visualized with DAPI (blue). Magnification, ×200. Images were digitally cropped in Photoshop CS6.
To act as a receptor for CooP, MDGI should be expressed by the tumor cells and/or tumor-associated stromal cells. First, we investigated whether MDGI was expressed in those tumors to which the CooP peptide homed. FITC-labeled CooP peptide was injected intravenously into mice bearing intracranial HIFko (A–B) or U87MG (C–F) gliomas. CooP peptide and MDGI were visualized using antibodies against fluorescein (red in A, C, E, and F) and MDGI (green in B and D). Overexpression of MDGI in the U87MG gliomas (F) increased homing of the CooP peptide compared with the control U87MG tumors (E). Nuclei were visualized with DAPI. Magnification, 400 ×. Images were digitally cropped in Photoshop CS6.

To act as a receptor for systemically administered peptide, MDGI should be accessible via the circulation. To further validate MDGI as a receptor for the CooP peptide, we established an MDGI expressing U87MG glioma cell line and monitored whether the presence of MDGI would affect CooP homing to the intracranial tumors derived from MDGI expressing or control U87MG cells. MDGI expression substantially increased the peptide homing to these tumors (Fig. 2E and F).

**Circulating anti-MDGI antibody localizes to tumor-associated blood vessels**

To act as a receptor for a systemically administered peptide, MDGI should be accessible via the circulation. Therefore, we next injected goat anti-MDGI antibodies intravenously to U87MG tumor mice and investigated whether the antibodies could be detected in the tumor tissue 60 minutes after injection. Blood vasculature was visualized with anti-PECAM-1 staining. Staining of tissue sections from various organs with secondary antibodies showed accumulation of the anti-MDGI antibody in the tumor-associated vasculature (Fig. 3A), whereas blood vessels in other tissues such as muscle (Fig. 3B), heart (Fig. 3C), normal brain (Fig. 3D), kidney, liver, and lung (data not shown) showed no detectable accumulation of the antibody. Control goat IgG did not accumulate in the tumor or any other tissue examined (Fig. 3E–H). We also stained tissue sections for MDGI to detect expression of this protein in sites not accessible to the circulating antibody. In addition to the tumor (Fig. 3I), muscle (Fig. 3J) and heart (Fig. 3K) were also positive for MDGI expression. No MDGI expression was detected in normal brain with the antibody (Fig. 3L). Thus, only in tumor MDGI was accessible via the blood circulation and could therefore act as a specific receptor molecule for the intravenously administered homing peptide and anti-MDGI antibody.

**Peptide-based SPECT/CT imaging of brain tumors**

Next, we studied the potential of the CooP peptide in brain tumor imaging using SPECT/CT. We labeled the diethylene-triamine-penta-acetic acid conjugated CooP and injected it intravenously into mice bearing intracranial U87MG gliomas. Tumor sections were then studied for the presence of the peptide as well as for MDGI expression. MDGI was detected both in the U87MG and HIFko xenografts (Fig. 2B and D). Furthermore, partial colocalization of MDGI and the peptide was observed in the tumor tissue (arrows in Fig. 2A–D). Importantly, MDGI was expressed in all tumors the CooP peptide homed to (intracranial HIFko, U87MG, and BT4C tumors) and MDGI expression was absent in tumors to which the CooP peptide did not home (intracranial VEGF-overexpressing tumor, orthotopic MDA-MD-231 breast cancer xenograft, and subcutaneous HIFko tumor). Moreover, we stained two additional xenograft models of glioblastoma multiforme for the presence of MDGI. Intracranial tumors derived both from the murine EGFRvIII transformed, INK4/Arf−/− NSCGs and from a patient tumor (GBM43; ref. 18) expressed moderate levels of MDGI (Supplementary Fig. S2).

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2 hours after injection (Fig. 4C and D). Tumor site radioactivity was approximately 20-fold higher at 15 minutes after injection and about 12-fold higher at 2 hours after injection compared with the contralateral brain hemisphere, indicating marked CooP peptide accumulation in the malignant tissue (Fig. 4E). Importantly, only the tumors showed increased accumulation of the CooP peptide compared with the control peptide at 2 hours after injection (Fig. 4F).

CooP-targeted delivery of chemotherapeutics

Since CooP peptide homed to the brain tumor satellites, we wanted to study the potential of CooP-targeted therapy to treat invasive brain tumors. As a proof-of-concept, we covalently conjugated clorambucil (Cbl) to the CooP peptide. Since CooP was not taken up by the cells, a cell penetrating peptide sequence (CPP; ref. 23) was added between CooP and Cbl to facilitate conjugate internalization (Fig. 5A). On the basis of their infiltrative growth pattern, intracranial HIFko tumors were chosen as a model. We treated the intracranial HIFko tumor-bearing mice with intravenous injections of either saline, free Cbl, or CooP-CPP-Cbl (5 mg/kg, 4 mice/group) every second day starting on day 5 after implantation and monitored the weight of the animals during the study. At day 14, mice in the saline and free Cbl groups had lost more than 10% of their starting weight and had to be sacrificed (Fig. 5B). In the targeted therapy group, mice gained weight and did not show any signs of tumor burden indicating that the CooP-targeted drug treatment prolonged the life.
span of the animals (Fig. 5B). Quantification of tumor satellites in the brain showed significantly ($P = 0.045$) fewer satellites in the targeted drug treated animals than in the control groups (Fig. 5C), whereas no difference in the size of the primary tumor was observed (Fig. 5D).

MDGI expression in human brain tumors

Our results suggest that MDGI acts as an interacting partner for the CooP peptide in the experimental glioma models. Since MDGI expression in human brain tumors has not been reported earlier, we investigated the presence and localization of MDGI in clinical brain tumor samples by immunohistochemistry. First, we analyzed a panel of astrocyte-derived tumor samples from craniotomy patients. Normal human brain (Fig. 6A) and grade 1 astrocytomas (Fig. 6B) did not express MDGI at detectable levels. Interestingly, the majority of the grade 2 (60%; Fig. 6C) and grade 3 astrocytomas (80%; Fig. 6D) showed moderate levels of MDGI mostly in the vasculature and in perivascular compartment. Of glioblastomas, 17 of 18 (94%) expressed very high levels of MDGI. The expression was concentrated on perinecrotic areas of the tumors (Fig. 6E and F). Sixty-seven percent of ependymomas (Fig. 6G) were moderately positive while medulloblastomas (Fig. 6H) showed no MDGI expression. We also had access to a small tissue array of human glioblastomas ($n = 46$). In this analysis, 70% (32/46) of gliomas stained positive for MDGI. To investigate MDGI localization in gliomas more...
Peptide-targeted delivery of therapeutics prolongs the life-span of mice bearing intracranial HIFko tumors. A, internalization of fluorescein-labeled CooP, CPP, or Coop-CPP peptides. Fluorescence was measured at 494/518 nm after 1 hour of incubation of HIFko cells with 5 μmol/L fluorescein-labeled peptides. Values represent the mean of 4 replicates in one representative experiment. Error bars, ± SD. B, HIFko cells were injected intracranially into nude mice. Treatment was started after tumor establishment at day 5 after implantation (arrow). Mice were treated with intravenous injections of either saline (PBS), free Cbl, or targeted drug (CooP-CPP-Cbl) every second day. Weight of the animals was monitored. Experiment was stopped when criteria for sacrifice was met (>10% weight loss). Graph shows the normalized, mean body weight in each group during the experiment (± SEM). The mean weight on day 0 was set as 100%. C, brain sections of the treated mice were imaged and brain area occupied by tumor satellites was quantified from four sections/animal. Graph shows percentage of brain occupied by the tumor satellites (± SEM). D, brain sections of the treated mice were imaged and brain area occupied by the primary tumors was quantified from four sections/animal. Graph shows percentage of brain occupied by the primary tumors (± SEM). * P < 0.05 (Student t test).

Figure 5.

Carefully, we stained paraffin-embedded sections with antibodies against MDGI and an endothelial marker, PECAM-1. MDGI was detected in tumor cells (Fig. 6I–K) and in vascular structures, which were positive for PECAM-1 staining in subsequent sections (Fig. 6L–P). Double-labeling with fluorescently labeled anti-MDGI antibodies and PECAM-1 antibodies showed that both proteins were present in the same cells (Fig. 6Q). Thus, our results demonstrate that MDGI is present in human brain tumors in a grade-dependent manner, its expression positively correlates with the histologic grade of the tumor and it is expressed both in tumor cells and in blood endothelium.

Discussion

Here we describe identification of a novel homing peptide, CooP, which recognizes an epitope in certain brain tumor cells and tumor-associated endothelium. We provide evidence to the effect that mammary-derived growth inhibitor (MDGI), acts as an interacting partner for CooP in brain tumors, and show that the CooP peptide can be used in selective delivery of an imaging agent and chemotherapeutic into glioblastomas. Finally, we show that human glioblastomas, but not the human normal brain, express MDGI, the CooP receptor.

The CooP peptide appears to have a highly selective specificity as a homing peptide. Binding of the CooP phage to cells isolated from glioblastomas, tumor homing of this phage in vivo, and the accumulation of the CooP peptide in glioblastomas, but not in the normal brain or other normal tissues demonstrate tumor specificity of the peptide. Our results also suggest further layers of specificity. CooP did not recognize the vessels in HIFko tumors that had been engineered to express VEGF, and unlike the original tumors, were able to induce angiogenesis to provide the vasculature to sustain tumor growth. Also, an orthotopically grown breast cancer was negative for CooP homing. These results show that CooP does not recognize an angiogenesis-specific marker, like most of the currently known tumor-homing peptides do (reviewed in ref. 12). Tumor-homing peptides and vascular tumor markers that are tumor type–specific have been described (10, 24, 25). Moreover, CooP did not recognize HIFko tumors grown subcutaneously, suggesting a specificity for brain-derived tumor vasculature. Other than their proximity to tumor cells, there are no markers for vessels co-opted by tumors (as opposed to arising as a result of tumor-induced angiogenesis). Hence, it is difficult to prove that CooP specifically recognizes co-opted tumor vessels, but the features discussed above, particularly the lack of binding to angiogenic tumor vessels, strongly suggest that this is the case. No peptide with such specificity has been described before.

Several lines of evidence indicate that the MDGI/FABP3 is the receptor that is recognized by CooP and the selective expression of which endows CooP with the specific homing properties. First, a yeast-2-hybrid screen using the peptide sequence as bait yielded three different MDGI cDNAs suggesting that it binds to the CooP peptide. Second, MDGI overexpression increased binding of CooP to cells in vitro and homing to tumor xenografts in vivo. Third, circulating antibodies against MDGI accumulated in tumor tissue and were nondetectable in other tissues, indicating that MDGI is accessible through the circulation for intravenously injected ligands. It is noteworthy that MDGI is also expressed in heart and skeletal muscle as previously described (26) but, importantly, the vascular expression is restricted to the tumor tissue, allowing the tumor-specific homing of the peptide. Staining of human glioma samples with anti-MDGI and PECAM-1 antibodies confirmed the vascular expression of MDGI.
Fatty acid binding proteins (FABP) are primarily intracellular proteins, although membrane-associated forms have also been reported (27). These FABPs mediate the transport of fatty acids and other hydrophobic ligands to tissues (28). The extracellular/cell surface localization of intracellular proteins is not unusual in tumors; cell surface or plasma membrane localization in tumors has been reported previously for several intracellular proteins: e.g., GRP78 (29), NUCLEOLIN (30), ANNEXIN (31), and P32 (32).

Figure 6. MDGI is expressed in tumor cells and in blood endothelial cells, and its expression positively correlates with tumor grade in clinical human brain tumor samples. MDGI expression was studied in sections from normal brain (A), grade 1 (B), grade 2 (C), and grade 3 (D) astrocytomas, as well as in primary (E, I-P) and secondary (F) grade 4 glioblastomas, in ependymomas (G), and in medulloblastomas (H) with anti-MDGI antibodies using immunohistochemical stainings. MDGI and PECAM-1 are shown as red color and nuclei in blue. I, MDGI expression in tumor cells. J, subsequent section stained with anti-PECAM-1 antibodies. K and L, specificity of the MDGI staining was shown by using rabbit IgG staining as a control for panels I and M. M and P, MDGI expression in PECAM-1–positive endothelial cells. N, higher magnification of the boxed area in M. O, higher magnification of the boxed area in P. Q, human glioblastoma (grade 4) sections were stained with fluorescently labeled antibodies against MDGI (red) and PECAM-1 (green). Nuclei were visualized with DAPI (blue). Inset, higher magnification of the boxed area showing that MDGI and PECAM-1 are present in the same cells. Magnification A–K, ×400; M–Q, ×200. Images were digitally cropped in Photoshop CS6.
The role of MDGI in tumor progression is somewhat controversial and seems to vary among different cancer types. MDGI appears to be the only FABP that affects cell proliferation and differentiation. This function may be separate from its ligand-binding function since it can be mimicked by its C-terminal peptide, which cannot bind fatty acids (33, 34). In sporadic breast cancers, the genomic region coding for MDGI is often deleted and MDGI appears to be downregulated in breast cancer cell lines via hypermethylation (35, 36). Moreover, MDGI has been reported to inhibit the growth of MCF-7 breast cancer cells and reduce their tumorigenicity (37) as well as inhibit invasion and adhesion of MDA-MB-231 breast cancer cells (36).

On the other hand, ectopic MDGI expression was shown to render breast and lung cancer cells resistant to treatment with the anti-EGFR antibody, cetuximab (36). In small-cell lung cancer, MDGI expression is significantly higher in the highly aggressive cells than in their less aggressive subtype (38). In gastric carcinoma, MDGI expression is also associated with tumor aggressiveness, progression, and poor patient survival (39). Our data show association of increased MDGI expression with high grade of brain tumors and suggest MDGI as a novel marker for highly malignant brain tumors.

In contrast to antibodies, which have been traditionally used for the specific delivery of therapeutic agents to several tumor types (40–43), homing peptides are faster and less expensive to produce and nonimmunogenic (12, 44). Coupling of therapeutic agents with peptides has also been shown to enhance drug penetration into the target tissue (45). Because of their small size, peptides penetrate the blood–brain barrier better, enabling the targeted delivery of therapeutic agents directly into the tumor tissue. Therefore, utilization of homing peptides offers an attractive alternative for targeting solid tumors such as malignant gliomas, which adapt to novel antiangiogenic therapies by increased invasion and co-option of preexisting vasculature (45–50). Importantly, CoolP peptide-conjugated therapeutics reduced the number of tumor satellites in the brain, suggesting that CoolP-targeted therapies could be useful as drug carrier to ablate tumor cells in areas that cannot be surgically removed or reached by angiogenesis-based therapies. In addition, MDGI, the CoolP receptor, may be a useful target molecule for the development of new therapies against malignant gliomas.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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