A Proteomic Approach for the Identification of Vascular Markers of Liver Metastasis

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Abstract

Vascular proteins expressed at liver metastasis sites could serve as prognostic markers or as targets for pharmacodelivery applications. We employed a proteomic approach to define such proteins in three syngeneic mouse models of liver metastasis. Vascular structures were biotinylated in vivo by a terminal perfusion technique, followed by mass spectrometric analysis of accessible biotinylated proteins. In this manner, we identified 12 proteins for which expression was selectively associated with liver metastasis, confirming this association by tissue immunofluorescence or in vivo localization with radiolabeled antibodies. In summary, our findings identify vascular proteins that may have prognostic or drug-targeting use in addressing liver metastases, a common issue in many advanced cancers. Cancer Res; 70(1); OF1–10. ©2010 AACR.

Introduction

A promising approach to cancer therapy uses monoclonal antibody derivatives for the selective delivery of bioactive agents (e.g., full immunoglobulins for Fc-mediated cell killing, drugs with cleavable linkers, radionuclides, photosensitizers, procoagulant factors, and cytokines) to the tumor environment, thus sparing normal tissues (1–8). Distant site metastasis is the leading cause of cancer-associated mortality (9), thus the identification of selective and accessible markers of tumor metastasis represents an essential requirement for the development of antibody-based pharmacodelivery strategies capable of targeting disseminated lesions (10). Ligand-mediated pharmacodelivery options are particularly attractive in consideration of the fact that many conventional cytotoxic agents and therapeutic proteins typically exhibit a reduced uptake at the tumor site compared with normal organs (11, 12).

Our group has developed a chemical proteomics methodology for the identification of proteins which are readily accessible from the vasculature. The method relies on a covalent biotinylation of vascular proteins by the in vivo perfusion of laboratory animals (13, 14) or the ex vivo perfusion of surgically resected organs from patients (15, 16), using reactive ester derivatives of biotin. Biotinylated proteins can be efficiently recovered from normal tissues and pathologic specimens (e.g., tumors) by lysis in the presence of strong detergents, followed by capture on streptavidin-sepharose (14). On-resin tryptic digestion of biotinylated proteins, followed by nano-capillary high-performance liquid chromatography (HPLC) separation of eluted peptides and their identification and relative quantification in the presence of internal standards (17, 18), allows the characterization of atlases of vascular proteins in normal organs and at sites of disease, thus facilitating the discovery of accessible markers of pathology.

In this study, we report the results of a perfusion-based chemical proteomics study performed on three different syngeneic mouse models of liver metastasis. M5076 is a murine reticulum cell sarcoma originated spontaneously in the ovary of C57BL/6 mice and is highly invasive and metastatic. When transplanted subcutaneously into mice, M5076 forms tumors that spontaneously metastasize to the liver (19, 20). Colon38 and SL4 are murine colon carcinomas metastasizing to the liver thereby mimicking the metastatic spread of colorectal cancer in humans. SL4 has a higher metastatic potential and was established by Morimoto-Tomita and colleagues by repeated cycles of in vivo selections (21).

Among the 712 proteins identified with at least two peptides, 120 displayed a preferential expression in liver with metastasis. For 12 of 12 of these markers, which were overexpressed in at least two different models of metastasis and for which antibodies were available, a preferential expression in metastatic lesions was confirmed by immunofluorescence analysis. Furthermore, radiolabeled preparations of monoclonal antibodies specific to periostin, to the extra-domain A (EDA) of fibronectin and to angiopeptin-related protein 2, were shown to selectively target liver metastasis in vivo, following i.v. administration.

Materials and Methods

Animals. Procedures involving animals and their care were conducted in conformity with the institutional guidelines...
that are in compliance with national and international laws and policies. Female C57BL/6 mice were obtained from Harlan and used at 5 to 6 wk of age.

**Cell lines and cell culture.** Mouse colon carcinoma cell lines, Colon38 and SL4, were kindly provided by Dr. Tsatsuro Irimura (University of Tokyo, Tokyo, Japan). Cells were maintained in a 1:1 mixture of DMEM and Ham’s F-12 medium (Life Technologies) containing 10% heat-inactivated FCS. Mouse reticulum cell sarcoma M5076 (22) were maintained in RPMI 1640 containing 15% heat-inactivated horse serum (Invitrogen Life Technologies).

**Tumor models.** Liver metastasis were obtained by intrasplenic injection of 1 × 10⁶ cells in 50 μL of suspension (Colon38 and SL4; ref. 21) and by subcutaneous injection of 1 × 10⁶ cells in 200 μL of suspension (M5076; ref. 23). Tumor-bearing mice were used for in vivo biotinylation, targeting experiments, and organ excision for immunohistochemical analysis.

**In vivo biotinylation.** In vivo biotinylation experiments were performed as previously described (13, 14). After perfusion, organs and tumors were excised and specimens were either freshly snap-frozen for preparation of organ homogenates or embedded in cryoembedding compound (Thermo Fisher Scientific) and frozen in liquid nitrogen–cooled isopentane for the preparation of cryosections for histochemical analysis. A total of 38 tumor-bearing mice (Colon38, SL4, and M5076) and 11 healthy mice were perfused. Unperfused mice (healthy and tumor-bearing) were used as negative controls for the proteomic analysis.

**Preparation of protein extracts for proteomic analysis.** Livers (with or without metastases) were resuspended in lysis buffer [2% SDS, 50 mmol/L Tris, 10 mmol/L EDTA, and Complete Protease Inhibitor Cocktails (Roche Diagnostics)] in PBS (pH 7.4), using 40 μL/mg of tissue. The specimens were then homogenized using an Ultra-Turrax T8 disperser (IKA-Werke) and sonicated using a Vibra-cell (Sonics), followed by 15 min of incubation at 80°C and 20 min of centrifugation at 15,000 × g. The resulting supernatants ("total protein extracts") were used for the subsequent capture step on streptavidin-resin, determining protein concentration with the BCA Protein Assay Kit (Thermo Fisher Scientific).

**Purification of biotinylated proteins.** For each sample, 400 μL streptavidin-sepharose (GE Healthcare) slurry were washed thrice in buffer A (1% NP-40, 0.1% SDS in PBS), pelleted, and mixed with 5 mg of total protein extract. The capture of biotinylated proteins was allowed to proceed for 2 h at room temperature in a revolving mixer. The supernatant was removed, and the resin was washed thrice with buffer A, twice with buffer B (0.1% SDS, 2 mol/L NaCl in PBS, at 40°C), and eight times with digestion buffer (50 mmol/L Tris-HCl, 1 mmol/L CaCl₂, pH 8.0). Finally, the resin was resuspended in 200 μL of digestion buffer and 20 μL of sequencing grade-modified porcine trypsin (stock solution of 80 ng/μL in digestion buffer; Promega) were added. Peptide digestion was carried out overnight at 37°C under constant agitation. Peptides were desalted, purified, and concentrated with C18 microcolumns (OMIX tips; Varian, Inc.). After lyophilization, peptides were stored at −20°C.

**Nanocapillary reverse-phase HPLC with online fraction spotting onto matrix-assisted laser desorption/ionization plates.** Tryptic peptides were separated by reverse-phase HPLC using an UltiMate nanoscale LC system and a FAMOS microautosampler (LC Packings) controlled by the Chromeleon software (Dionex). Mobile phase A consisted of 2% acetonitrile and 0.1% trifluoroacetic acid in water, mobile phase B of 80% acetonitrile and 0.1% trifluoroacetic acid in water. The flow rate was 300 nL/min. Lyophilized peptides derived from the digestion of biotinylated protein affinity purified from 2 mg of total protein were dissolved in 5 μL of buffer A and loaded on the column (inner diameter, 75 μm; length, 15 cm; filled with C18 PepMap 100, 3 μm, 100 Å beads; LC Packings). The peptides were eluted with a gradient of 0% B for 3 min, 0% to 52% B for 200 min, 52% to 100% B for 10 min, 100% B for 5 min, and the column was equilibrated with 100% A for 20 min before analyzing the next sample. Eluting fractions were mixed with a solution of 3 mg/mL a-cyano-4-hydroxycinnamic acid, 277 pmol/mL of each of the four internal standard peptides ([des-arg⁹]-bradykinin, neurotensin, angiotensin I, and adrenocorticotropic hormone fragments 1–17; all from Sigma), 0.1% trifluoroacetic acid, and 70% acetonitrile in water and deposited on a blank matrix-assisted laser desorption/ionization (MALDI) target plate (832 spots per sample) using an online Probot system (Dionex). The final concentration of each internal standard peptide was 50 fmol per spot.

**MALDI-time of flight/time of flight mass spectrometry.** MALDI-time of flight (TOF)/TOF analyzer was carried out with the 4800 MALDI TOF/TOF Analyzer (Applied Biosystems). All spectra were acquired with a solid-state laser (355 nm) at a laser repetition rate of 200 Hz. After measuring all samples in the mass spectrometry (MS) mode, a maximum of 15 precursors per spot were automatically selected for subsequent CID fragmentation. Resulting spectra were processed and analyzed using both the ProteinPilot software (Paragon Algorithm, Applied Biosystems) and the Global Protein Server Workstation [Applied Biosystems, using internal MASCOT (Matrix Science, London, UK)], for matching MS and MS/MS data against databases of in silico–digested proteins. The data obtained were screened against a database of all mouse proteins downloaded from the European Bioinformatics Institute homepage. Furthermore, the following analysis settings were used for the identification of peptides and proteins: (a) precursor tolerance, 15 ppm; (b) MS/MS fragment tolerance, 0.2 Da; (c) maximal missed cleavages, 2; and (d) one variable modification (oxidation of

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methionine). Peptides were considered correct calls when the confidence interval was >95%.

**Relative protein quantification by DeepQuanTR software.** The DeepQuanTR software has been described in detail elsewhere. Briefly, after MS acquisition, data related to the individual peaks (fractions, intensities, m/z ratios) were loaded into the DeepQuanTR software, which performed a normalization of individual signal intensities to the internal standard peptides and an annotation (peptide identification and association with a parent protein). Normalized intensities for the individual peptides from all samples of each liver group (i.e., healthy, M5076, SL4, and Colon38 mice) were used for the computation of DeepQuanTR peptide and protein scores, indicating the relative abundance of individual peptides and proteins in the various groups of samples.

**Immunofluorescence analysis.** All immunofluorescence analyses were performed according to standard protocol on aceton-fixed cryostat sections (10 μm) of freshly frozen liver metastases (Colon38, SL4, and M5076). Staining with anti-embigin, anti-fibulin-2, anti-latent transforming growth factor β-binding protein 4 (LTBP-4), and anti-orsomucoid 1 antibodies were done using goat anti-rabbit Alexa Fluor-594 as secondary antibody. Donkey anti-goat Alexa Fluor-594 was used as a secondary antibody for the detection of apolipoprotein H, inter-α-trypsin inhibitor heavy chain 4, and versican. Staining with anti-angiopoietin-related protein 2 and anti-periostin was obtained with donkey anti-rat Alexa Fluor-488. Immunofluorescent staining with scFv antibodies was counterstained with 4′,6-diamidino-2-phenylindole. Slides were mounted with fluorescent mounting medium (DakoCytomation) and examined by an Axioskop2 microscope (Zeiss).

**Biodistribution studies.** *In vivo* targeting performance of four monoclonal antibodies against oncofetal fibronectin, periostin, angiopoietin-related protein 2, and an antibody of irrelevant specificity was evaluated by quantitative biodistribution analysis as previously described (24). Mice bearing Colon38 liver metastases were sacrificed 48 h after i.v. injection of 125I-labeled antibodies. Organs were weighed and radioactive content was counted with a Cobra γ-counter (Packard).

**Radioluminography (phosphor image analysis).** To investigate the distribution of radiolabeled antibodies within the tumor mass, both whole metastases-bearing lobes and 20-μm sections were analyzed with a phosphor storage plate. Whole lobes were resected, photographed, and exposed overnight at 4°C to the plate. Sections were aceton-fixed, stained with hematoxylin, scanned using a desktop scanner to correlate the morphology with the antibody distribution, and exposed to the phosphor plate for 14 d. Phosphor storage plates were scanned using a BAS-5000 phosphor image reader (FujiFilm).

**Microautoradiography.** Forty-eight hours after the i.v. injection of radiolabeled antibodies, mice were sacrificed and metastatic livers were embedded and frozen in optimal cutting temperature compound medium (Thermo Fisher Scientific). Ten-micrometer sections were cut, fixed with ice-cold acetone, and coated with NBT Kodak autoradiography emulsion (Kodak). After drying, the slides were stored at 4°C in the dark for ~4 wk. The autoradiography emulsions were developed (Kodak Developer D-19) for 4 min and fixed (Kodak Eastman Fixer) for 5 min. Finally, slides were rinsed with deionized water and counterstained with hematoxylin (Sigma).

**Immunofluorescence analysis of *in vivo*–injected antibodies.** To investigate the *in vivo* distribution and penetration of the antibodies within the metastases, immunofluorescence staining was performed 48 h after i.v. injection. Fixed sections (10 μm) were incubated with hamster anti-mouse CD31 antibody and staining was obtained using donkey anti-rat Alexa Fluor-488 and goat anti-hamster Cy5 secondary antibodies. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Slides were mounted with fluorescent mounting medium (DakoCytomation) and analyzed with a confocal laser scanning microscope (LSM 510 META; Zeiss). Images were further processed using ImageJ software. To conform with previous stainings, Alexa Fluor-488 is shown in red and Cy5 in green.

**Results**

Liver metastases of Colon38, SL4, and M5076 tumors were developed in C57BL/6 immunocompetent mice by subcutaneous (M5076) or intrasplenic (Colon38 and SL4) injection of tumor cells. Figure 1 shows a schematic representation of the perfusion procedure used for *in vivo* biotinylation. Following terminal anesthesia, the thorax was opened and the large circulation perfused by intracardiac administration of ~17 mL of an aqueous solution of Sulpho-NHS-LC-Biotin (1 mg/mL) over 5 min, followed by perfusion with a quenching solution to block unreacted biotin ester. In this procedure, proteins are biotinylated not only on endothelial cells but also in the perivascular extracellular matrix and on the membrane of perivascular cells (10, 13). A total of 49 mice were perfused, including 11 healthy mice, whose normal livers served as negative controls for the identification of markers of metastasis. Furthermore, nonperfused mice (healthy and tumor-bearing) were used to evaluate nonspecific binding of proteins to the streptavidin resin. Liver metastases of M5076 were typically smaller and more numerous compared with Colon38 and its highly metastatic variant SL4. In all cases, however, an efficient biotinylation not only of liver vessels and sinusoids but also of vascular structures in metastatic lesions could be confirmed by streptavidin-based staining of liver sections (Fig. 1B).

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in vivo biotinylation, normal and metastatic livers were processed separately, homogenized in the presence of 2% SDS and submitted to chromatography on streptavidin-sepharose. After extensive washing, biotinylated proteins were tryptically digested on-resin. The resulting peptides were separated by reverse-phase nano-HPLC, mixed with pure peptides serving as internal standards for relative quantification (17, 25), and analyzed by MALDI-TOF and MALDI-TOF/TOF (Fig. 1A).

We used DeepQuanTR for the identification of regulated proteins in the various liver specimens. This software package allows the pairwise comparison of the average normalized signal intensities for the multiple tryptic peptides corresponding to the same protein in the various liver specimens. Figure 2 depicts the DeepQuanTR-computed average values for some of the most relevant regulated proteins found in the study. Proteins upregulated in metastatic liver compared with the healthy organ were displayed in green,

Figure 1. In vivo biotinylation of proteins accessible from the vasculature. A, schematic representation of the chemical proteomics approach for target discovery. Healthy mice (n = 11) or mice bearing Colon38 (n = 11), SL4 (n = 14), or M5076 (n = 13) metastases were subjected to the terminal perfusion with a reactive ester derivative of biotin. This procedure resulted in the covalent modification of proteins accessible from the bloodstream (e.g., luminal and abluminal membrane proteins of endothelial cells, proteins of the extracellular matrix, and proteins on the membrane of cells in close proximity to blood vessels) with biotin. Subsequently, tissue specimens from the normal liver of healthy mice as well as from the metastases were used for the preparation of total protein extracts. Biotin-tagged proteins were then enriched on streptavidin sepharose and digested on-resin with trypsin. The resulting peptides were separated by nano-capillary reverse-phase HPLC and eluting fractions were mixed with constant amounts of internal standard peptides. Following MALDI-TOF and MALDI-TOF/TOF MS analysis of each fraction, the DeepQuanTR software was used for the relative quantification of protein abundances. B, representative liver metastases of analyzed tumor models and histochemical staining of biotinylated structures. Cryosections from the different liver metastasis models were stained with streptavidin-alkaline phosphatase and Fast Red, demonstrating efficient and selective biotinylation of vascular structures in the metastases. Bars, 100 μm.
whereas downregulated proteins were displayed in red. The color intensity relates to the magnitude of the differences in MS signals for the various peptides (see also Fig. 3). Relevant proteins include embigin, splice variants of fibronectin and of tenascin-C, fibulin-2, LTBP-4, periostin, versican, vitronectin, CD44, CD93, CD98, and thrombospondin-1. The complete list of regulated proteins can be found in Supportive Table S1.

We focused our attention on a subset of proteins for which antibodies were available either commercially or in our laboratory. Figure 3 shows the relative average signal intensities for multiple individual tryptic peptides belonging to 12 individual proteins, which have been studied in more detail. Most proteins exhibited an upregulation in all three models of liver metastasis. However, integrin α-M exhibited a striking overexpression only in the M5076 model, in line with the macrophage origin of this tumor (19). By contrast, orosomucoid-1 and splice isoforms of tenascin-C were found to be upregulated mainly in the liver metastases of colorectal cancer origin. The alternatively spliced fibronectin domains EDA and IIICS exhibited a dramatic upregulation in metastatic liver. By contrast, unspliced fibronectin portions could be detected both in normal liver and in metastatic lesions (Supportive Fig. S1). The immunofluorescence analysis (Fig. 3) nicely confirmed the protein expression data derived from the MS analysis and presented either as global DeepQuanTR values for the individual gene products (Fig. 2) or as patterns of average signal intensities for the individual tryptic peptides (Fig. 3). Some of the strongest vascular staining patterns were observed for angiopeptin-related protein 2, fibulin-2, LTBP-4, inter-α-trypsin inhibitor heavy chain 4, periostin, as well as for splice isoforms of fibronectin and of tenascin-C (Fig. 3). Versican displayed a clear, yet weaker neovascular staining and has recently been associated with the metastatic phenotype by macrophage activation (26).

To confirm that proteins overexpressed at metastatic sites could be targeted in vivo using specific monoclonal antibodies, we performed biodistribution studies, autoradiographic analyses, and microscopic staining using radioiodinated

![Figure 2: DeepQuanTR output and regulation of selected proteins. A, total list of regulated proteins. Protein abundances of four Colon38, five SL4, and five M5076 samples are displayed with a color code related to the corresponding DeepQuanTR score (maximum = e^6-fold upregulation compared with the healthy liver group; minimum = e^6-fold downregulation). Green, proteins upregulated in metastatic liver compared with healthy liver; red, downregulated proteins. The list presented was sorted according to average relative protein abundances. B, regulation of selected proteins. The regulation of 26 proteins indicated with gene name, primary SwissProt accession number, and number of identified peptides. Gene names in boldface indicate proteins that were further investigated. Cd44 (CD44), Fn (fibronectin), Vtn (vitronectin), Cd93 (CD93), Thbs1 (thrombospondin-1), Emb (embigin), ApoH (apolipoprotein H), Angptl2 (angiopoietin-related protein 2), Tnc (tenascin-c), Fhbn2 (fibulin 2), Ilt3 (inter-α-inhibitor heavy chain 3), Lrg1 (leucine-rich α2-glycoprotein), ApoA4 (apolipoprotein A-IV), Hp (hemopexin), Ilgam (interleukin α-M), Ltbp-4, Cd98 (CD98), Col12a1 (collagen α-XII), Ilth4 (inter-α-trypsin inhibitor heavy chain 4), Pxdn (peroxidasin homologue/MG-50), Vcan (versican), Siglec1 (sialoadhesin), Postn (periostin), and Orm1 (orosomucoid 1).](#)
Figure 3. Peptide regulation and target validation by immunofluorescence. Left, peptide DeepQuanTR values for up to six peptides per protein. Representative peptides were chosen for proteins identified with more than six peptides. Peptides annotated to known splice isoforms are labeled. Right, immunofluorescence analyses for all three metastasis models. Proteins of interest (red) and nuclei (blue). Metastases are characterized by a more intense blue staining, which reflects the higher cellular density. There is a strong correlation between the identification of a protein regulation by DeepQuanTR and the corresponding immunofluorescence analysis (e.g., integrin α-M, a protein found to be highly upregulated only in liver metastases deriving from the M5076 reticulum sarcoma, shows an almost exclusive expression in this model). Furthermore, splice isoform–specific regulations have been identified (e.g., for the extra-domains A and IIICS of fibronectin, and for the large isoform of tenascin-c) and validated by immunofluorescence analysis. Bars, 200 μm. Proteins are indicated according to gene name; corresponding protein names are reported in Fig. 2.
Figure 4. **In vivo** tumor targeting results using radiolabeled antibody preparations in mice bearing Colon38 metastases. 

**A**, biodistribution results (expressed as %ID/g ± SE) of monoclonal antibodies specific to the EDA domain of fibronectin, periostin, and angiopoietin-related protein 2. A rat IgG of irrelevant specificity in the mouse was used as a negative control. LM, liver metastasis; ST, spleen tumor; HE, heart; IN, intestine; KI, kidney; LI, liver; LU, lung; MU, muscle; SP, spleen; BL, blood.

**B**, macroscopic autoradiographic analysis of antibody localization in liver metastases. A photograph of a metastatic liver lobe is presented alongside with the corresponding phosphor image autoradiogram. In addition, 20-μm-thick slides were stained by hematoxylin (blue) and submitted to phosphor image analysis.

**C**, microscopic analysis of antibody localization on metastatic lesions. Microautoradiograms of 20-μm-thick sections, counterstained with hematoxylin. Sections from the same specimens were also processed by immunofluorescence, staining for nuclei (blue), CD31 as a blood vessel marker (green), and the **in vivo** localized monoclonal antibody (red). Bars, 100 μm. Metastases are marked (M).
antibody preparations following i.v. administration. Among
the antigens exhibiting the most intense staining patterns
in immunofluorescence (Fig. 3), we chose splice variants of
fibronectin (27), peristin, and angiopoietin-related protein
2 as targets, for which good-quality monoclonal antibodies
were available (27). Figure 4A depicts biodistribution results
in liver metastases, primary spleen tumors, and normal or-\n\begingroup\setlength{\parindent}{0em}gan\endgroup\n\sloppy
\textit{in vivo} targeting results presented in this study with anti-EDA, anti-peristin, and anti-angiopoietin-related protein 2 antibodies were extremely promising, with tumor/blood ratios of >30 for primary spleen tumors and >15 for liver metastases, using the F8 anti-EDA antibody 48 h after i.v. administration (Fig. 4). Importantly, as the targeted structures represent only a small portion of the total tumor mass (34), the local concentration of antibody delivered to vascular tumor structures is particularly high and should facilitate pharmacodelivery applications.

The proteins identified in our analysis included antigens expressed by endothelial cells, by tumor cells, and in the extracellular matrix. Restricting the analysis to luminal antigens only would require the availability of biotinylation reagents that would not cross the endothelial layer. However, our group has had good experiences in tumor-targeting applications using monoclonal antibodies specific to stromal antigens and to components of the modified subendothelial extracellular matrix (1, 2). In particular, derivatives of monoclonal antibodies specific to the EDA (10, 27) and EDB domain of fibronectin (24, 35), and to the A1 domain of tenasin-C (36, 37), have exhibited excellent tumor-targeting properties in animal tumor models (37–40) and in patients with cancer (41, 42), and are currently being investigated in phase I and phase II clinical trials in oncology and in arthritis. The results described in this article indicate that a number of other antigens could be considered for \textit{in vivo} pharmacodelivery applications. Among them, peristin could be efficiently and selectively targeted by a cognate monoclonal antibody and thus deserves a closer investigation as an antigen for the development of ligand-based anticancer imaging and innovative therapeutic strategies.

\section*{Disclosure of Potential Conflicts of Interest}

No potential conflicts of interest were disclosed.
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