Visualizing cancer and response to therapy in vivo using Cy5.5-labeled factor VIIa and anti-tissue factor antibody

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Abstract
We have developed a specific technique for imaging cancer in vivo using Cy5.5-labeled factor VIIa (fVIIa), clotting-deficient FFRck-fVIIa, paclitaxel-FFRck-fVIIa, and anti-tissue factor (TF) antibody. FVIIa is the natural ligand for TF. We took advantage of the fact that vascular endothelial cells (VECs) in cancer, but not normal tissue, aberrantly express TF due to its induction by vascular endothelial growth factor (VEGF). Under physiological conditions, TF is expressed by stromal cells and outer blood vessel layers (smooth muscle and adventitia), but not by VECs. We hypothesized that labeled fVIIa or anti-TF antibodies could be used to image the tumor vasculature in vivo. To test this, Cy5.5-labeled fVIIa, FFRck-fVIIa, paclitaxel-FFRck-fVIIa, and anti-TF antibody were developed and administered to athymic nude mice carrying xenografts including glioma U87EGF-fVIIa, pancreatic cancer ASPC-1 and Mia PaCa-2, and squamous cell carcinoma KB-V1. Cy5.5 labeled with these targeting proteins specifically localized to the tumor xenografts for at least 14 days but unconjugated Cy5.5 did not localize to any xenografts or organs. This method of imaging TF in the tumor VECs may be useful in detecting primary tumors and metastases as well as monitoring in vivo therapeutic responses.

Introduction
Tissue factor (TF) is a 47 kDa transmembrane glycoprotein and the cognate receptor of coagulation factor VII/VIIa (fVIIa). This ligand/receptor protein–protein interaction enjoys the highest affinity and specificity of all such pairings. Under physiological conditions, TF is expressed by stromal cells and outer blood vessel layers (smooth muscle and adventitia), but not by vascular endothelial cells (VECs) [1–3]. Injury of the vascular wall causes a TF to bind to fVIIa in the plasma, initiating thrombosis and leading to thrombin/fibrin deposition and hemostasis.

The full length TF (fTF) gene is divided into six exons, whereas in alternatively spliced TF (asTF), exon 5 is absent, resulting in a truncated extracellular domain. Activation of fTF by fVIIa leads to signaling through protease activated receptor-2 (PAR-2), resulting in angiogenesis and tumor growth, and through PAR-1, resulting in coagulation. The absence of a transmembrane domain causes asTF to be soluble and present in the circulation, yet, asTF is capable of adhering to β1 integrin and adhering to cells. By this mechanism, it retains the ability to bind fVIIa and trigger the TF-fVIIa-induced coagulation cascade, in some cases leading to venous thromboembolic complications [4–6].

Aberrant expression of TF occurs both in cancer cells and in VECs, the latter due to induction by cancer-secreted VEGF [7–11], and these mechanisms account for the increased incidence of thrombosis observed in cancer patients (Trousseau sign) [12–15]. A direct correlation between TF expression and histologic grade has been noted for multiple tumor types, including gliomas [12], colorectal cancer [13], pancreatic cancer [14,15], breast cancer [16,17], ovarian cancer [17] and lung cancer [17].

The high affinity of TF-fVIIa binding and the specificity of endothelial TF expression in neoplastic tissue provide an excellent opportunity to target the vasculature of TF-expressing tumors using fVIIa as a targeting ligand for imaging and drug delivery. Indeed, we have previously coupled both the potently cytotoxic curcumin analogue EF24 [18] and paclitaxel with fVIIa, and found that upon binding of fVIIa...
to TF, receptor-mediated endocytosis brings the drug-conjugate inside target breast cancer cells, where enzymatic cleavage releases the cytotoxic agent [18]. A major challenge in using fVIIa clinically is the risk of activation of the coagulation cascade by the binding of TF to fVIIa. An enzymatically inactivated form of fVIIa was synthesized by conjugating phenylalanine–phenylalanine–arginine chloromethyl ketone (FFRck) to its active site in the serine protease domain [19]. The resulting FFRck-fVIIa is unable to initiate blood clotting, yet has a 5-fold greater binding affinity to TF than native fVIIa [20]. We have conjugated EF24 and paclitaxel to FFRck-fVIIa, resulting in the EF24-FFRck-fVIIa [18,21] and paclitaxel-FFRck-fVIIa conjugates, respectively [22]. These drug-conjugates demonstrated cytotoxic activity against the tumor vasculature in animal xenografts. EF24-FFRck-fVIIa inhibited the growth of subcutaneous breast cancer xenografts and paclitaxel-FFRck-fVIIa suppressed the growth of breast cancer xenografts in the lung [23]. One possible advantage of these two conjugates is their lack of binding to the vascular endothelium of adjacent normal tissues that lack TF expression. FFRck-fVIIa is safe and already in clinical use, so its conjugation to a cytotoxic agent is rational [24,25]. Since the drug-conjugate specifically delivers drug to target cells where TF is concentrated, this approach achieves a cytotoxic effect at a lower drug concentration than required for unconjugated drug.

Several novel cancer agents target the tumor vasculature and angiogenesis, and animal models provide an essential component of the screening and development of such new cancer drugs. The development of a reliable imaging modality for in vivo evaluation of the tumor vasculature is an important step in facilitating this process. Targeting TF for imaging may provide a non-invasive and cost effective method to evaluate the tumor vasculature in animal models.

Cyanine dye, Cy5.5 NHS ester, is a reactive agent for the labeling of amino-groups in peptides, proteins, and oligonucleotides. Cy5.5 is a far-red (and near-infrared) emitting dye which is ideal for fluorescence measurements where background fluorescence is a concern. It is also suitable for in vivo imaging experiments. An important aspect of molecular imaging is the ability to examine and quantify treatment responses in vivo by monitoring specific primary molecules or downstream targets. Cy5.5 is cost-effective and its labeling chemistries are easy to perform, making it suitable for potential anti-cancer drug development.

The objective of the current study is to evaluate the use of Cy5.5 conjugated with fVIIa, FFRck-fVIIa, paclitaxel-FFRck-fVIIa and anti-TF antibody as a modality to image the tumor vasculature in animal xenograft models.

Materials and methods

Materials

Cy5.5 mono-reactive NHS ester (10 mg) was purchased from Amersham, GE Healthcare Factor (Buckinghamshire, UK). Factor VIIa, phenylalanine–phenylalanine–arginine chloromethyl ketone conjugated to factor VIIa (FFRck-fVIIa, the active site-inactivated factor VIIa, abbreviated as ASIS) and a competitive inhibitor of fVIIa were provided by Dr. Lars C. Petersen, Novo Nordisk, Bagsvaerd, Denmark. Anti-TF antibody (Cat. No. 4501, 1 mg/mL) was purchased from American Diagnostica Inc., Stamford, CT.

Cell lines and animals

Mia PaCa-2 and ASPC-1 pancreatic cancer cells were purchased from the ATCC. U87EGFRviii glioma cells were provided by Dr. Daniel J. Brat. KB-V1 cervical squamous cell carcinoma (SCC) cells were from Dr. Dong M. Shin at Emory University. Athymic nude mice (nu/nu) were purchased from Harlan (Indianapolis, IN).

Conjugation of Cy5.5 with factor VIIa, anti-TF antibody, FFRck-fVIIa and paclitaxel-FFRck-fVIIa

Factor VIIa (5 mg/mL), FFRck-fVIIa (ASIS, Batch NLD013: 7 mg/mL), and anti-TF antibody (1 mg/mL) were dissolved in distilled water and dialyzed in 2 L of 0.1 M Na-carbonate buffer (pH 8.8) for 48 h. Cy5.5 (10 mg) was dissolved in 3 mL of 100% DMSO. An aliquot of Cy5.5 was added to the following proteins in approximately the indicated Cy5.5: protein ratios: fVIIa (1.5:1), FFRck-fVIIa (2:1), paclitaxel-FFRck-fVIIa (2:1) and anti-TF antibody (2:1), based on calculations following the manufacturer’s instruction. The mixtures were stirred gently for 1–1.5 h at room temperature. The resulting Cy5.5-protein conjugates were separated from unconjugated Cy5.5 by a Sephadex G25-150 column previously equilibrated with 0.1 M Na-carbonate buffer (pH 8.8). In a typical experiment, 1.8 mg of fVIIa in 0.6 mL in 0.1 M sodium-bicarbonate buffer, pH 8.8 was incubated with 1 mg of Cy5.5 mono-NHS ester in DMSO in 0.3 mL at room temperature for 1 h. Cy5.5-fVIIa and free Cy5.5 dye were separated using the Sephadex G25-150 column (8 mL). 0.3 mL (0.324 mL) of each fraction was collected (1 drop = 54 μL) for fractions 2–6, containing Cy5.5-fVIIa. Then fractions 7–14 with no color were eluted at 1 mL/fraction. Free Cy5.5 dye was eluted from fractions 15–21 and thereafter. Absorbance reading at A280 and A678 identified fractions containing Cy5.5-fVIIa (protein) and free Cy5.5 dye (no protein). Fractions with higher protein were determined using a Micro BCA protein assay kit (Pierce, Rockford, IL) and pooled. The protein concentration of the pooled fraction (1 mL total volume) typically was 0.7 mg/mL. The Cy5.5 to fVIIa ratio was calculated as 1.24:1, using the extinction coefficient 1.7 × 10^5 M⁻¹cm⁻¹ for the antibody, as determined by following the manufacturer’s manual.

Cell culture

U87EGFRviii glioma cells, Mia PaCa-2, and ASPC-1 pancreatic cancer cells were maintained in Eagle’s MEM medium with Earle’s salt, Dulbecco’s MEM and RPMI-1640 medium, respectively, containing 10% heat inactivated FBS, penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM l-glutamine, 0.1 mM non-essential amino acids at 37 °C and 5% CO2/95% air in a humidified atmosphere. The multidrug-resistant KB-V1 cervix squamous carcinoma cells (SCC) were
cultured in 85% Dulbecco’s MEM + 15% FBS + 200–1000 ng/mL vinblastine.

Immunohistochemistry for tissue factor

Archived surgically resected GBMs (6 cases) were obtained from Emory University Hospital Department of Pathology. Specimens were chosen based on the diagnosis and no other clinical identifiers were included. Formalin-fixed paraffin-embedded sections were deparaffinized and subjected to heat-induced epitope retrieval by steaming for 15 min. For TF IHC, slides were incubated with primary antibody (mouse monoclonal, 1:100; American Diagnostica, Stamford, CT) at 4°C overnight. Avidin–biotin–peroxidase complex was used to detect the antibodies using 3,3′-diaminobenzidine (DAB) as the chromogen. Standard positive controls and normal sera without primary antibodies as negative controls were used throughout. Staining intensity of TF in endothelial cells was evaluated within neoplastic tissues and within the adjacent normal brain tissue that was part of the surgical resection specimen.

ELISA assays for TF

TF and VEGF ELISA assay kits were purchased from American Diagnostica (Stamford, CT) and R&D Systems (Minneapolis, MN), respectively. The assays were performed as we have previously described for a variety of breast cancers and malignant melanomas [17,26].

Subcutaneous inoculation of cancer cells

U87EGFRviii glioma cells, MiaPaCa-2 and ASPC-1 pancreatic cancer cells at 10⁶ cells/0.1 mL, and KB-V1 SCC cells at 3 × 10⁶ cells/0.1 mL, were inoculated subcutaneously suspended in PBS. An aliquot of Cy5.5-FFRck-fVIIa or unconjugated Cy5.5 containing approximately 0.03 mg of Cy5.5/0.1 mL/mouse was injected intravenously into the lateral tail vein of athymic nude mice when all tumors reached 0.5–1.0 cm in diameter.

Imaging Cy5.5 near infrared in vivo

Imaging of Cy5.5-labeled fVIIa, FFRck-fVIIa, paclitaxel-FFRck-fVIIa and anti-TF antibody was monitored over time by detecting Cy5.5 in the whole animal according to the instructions of the IVIS Lumina Imaging System 100 Series (Xenogen, Alameda, CA). Standard filter set pairs for Cy5.5 were selected in the Filter Lock box and ensured that the excitation (615–665 nm) and emission (695–770 nm) filters were properly paired for Cy5.5. Imaging was carried out daily for up to 26 days after the injection (Figures 2–5). Mice were anesthetized by an intraperitoneal injection of the mixture of ketamine (50 mg/mL), xylazine (20 mg/mL) and sterile distilled water mixed at a ratio of 8, 1 and 9 volumes according to the IACUC approved protocol at Emory University. In Figure 5, tumors and normal organs were individually dissected and imaged. Cy5.5 was imaged at 2 days after the i.v. injection using the IVIS Imaging System 100 Series located in the Department of Animal Facility according to the manufacturer’s instructions.

Results

Immunohistochemical staining and ELISA assay of tissue factor of GBMs

VECs in breast and lung cancers are known to express TF [16,17]. To explore the hypothesis that VEGF secreted in any cancer induces TF expression in tumor VECs, we examined TF in GBM and normal brain tissues, since a vicious cycle of thrombosis due to TF/fVIIa interaction in VECs, necrosis and VEGF secretion in GBM is a typical phenomenon. Our studies of TF expression in specimens of non-neoplastic human brain and gliomas of increasing grades demonstrated that TF is expressed by endothelial cells in the innermost layer of blood vessels in human malignant gliomas, but not normal brain (Figure 1). IHC staining of TF in GBMs was performed as described previously [17]. We measured levels of TF in a panel of GBM tissues from Dr. Sarkaria of the Mayo Clinic determined using ELISA assay (Table 1) [27].

Cy5.5-labeled fVIIa and anti-TF antibody bind to TF expressed in VECs in human GBM U87MG-EGFRviii cells in vitro and in vivo

We next tested whether VECs in GBM xenografts express TF by using Cy5.5-labeled fVIIa and anti-TF antibody. We labeled fVIIa and anti-TF antibody with the fluorescent tag Cy5.5 and exposed U87MG-EGFRviii glioma cells to the Cy5.5-labeled-fVIIa and Cy5.5-labeled-anti-TF antibody in vitro and in vivo. In order to demonstrate the specificity of fVIIa binding to TF, unconjugated fVIIa was incubated in 5-fold excess with Cy5.5-labeled fVIIa in vitro. The resulting images revealed that the majority of the signal from the Cy5.5-labeled-fVIIa was reduced by the addition of excess...
unlabeled-fVIIa in vitro, indicating specificity of binding (Figure 2a). Consistent with these findings, we demonstrated a high degree of binding of both Cy5.5-labeled-fVIIa and Cy5.5-labeled-anti-TF antibody to U87MG-EGFRviii xenografts (Figure 2b), suggesting that fVIIa and the anti-TF antibody are able to bind TF expressed on VECs in glioma xenografts.

Cy5.5-labeled FFRck-fVIIa binds to TF expressed in VECs in pancreatic cancer xenografts

We used two pancreatic cancer models to test the hypothesis that TF is expressed in VECs of human pancreatic cancer. ASPC-1 cells express both fTF and aTF, while Mia PaCa-2 cells express neither fTF nor aTF. We predicted that Cy5.5-FFRck-fVIIa will specifically bind TF in tumor VECs of both tumor xenografts, since VEGF will induce TF in the tumor VECs even in Mia PaCa-2 xenografts lacking TF. We also wanted to test the duration and stability of the binding to be able to follow the therapeutic response. Cy5.5-FFRck-fVIIa and Cy5.5 dye alone were injected intravenously (i.v.) into athymic nude mice (nu/nu) bearing TF-expressing ASPC-1 and non-TF expressing Mia PaCa-2 pancreatic tumor xenografts on the right flank.

The specific localization of Cy5.5-FFRck-fVIIa with TF in the VECs in pancreatic tumor xenografts was observed from day 1 to day 26 in both ASPC-1 (Figure 3) and Mia PaCa-2 (Figure 4) tumors, whereas no specific localization of unconjugated Cy5.5 alone was observed.

Table 1. Tissue Factor (TF) levels in a panel of GBM tissues.

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Figure 2. (a) In vitro fluorescence imaging of Cy5.5 labeled fVIIa and Cy5.5-labeled anti-TF antibody with U87MG-EGFRviii cells. (b) In vivo imaging of U87-EGFRvIII (arrows) using Cy5.5-anti-TF antibody and Cy5.5-factor VIIa.
In vivo distribution of Cy5.5-labeled paclitaxel-FFRck-fVIIa

Thus far, we have seen the specific localization of Cy5.5-fVIIa and Cy5.5-anti-TF antibody in U87MG-EGFRviii glioma xenografts (Figure 2b) and of Cy5.5-FFRck-fVIIa in TF-expressing ASPC-1 and non-TF expressing Mia PaCa-2 (Figures 3 and 4) pancreatic tumor xenografts. In the latter experiment, fluorescence in a few non-tumor sites became visible after the injection of Cy5.5-FFRck-fVIIa during day 9 to day 26 in ASPC-1 xenografts (Figure 3), and day 19 to day 26 in Mia PaCa-2 xenografts (Figure 4). Fluorescence was visible possibly in the kidney regions of mice carrying ASPC-1 xenografts following the injection of Cy5.5 dye alone during days 1–3, then faded away, but this was not observed in mice carrying Mia PaCa-2 xenografts. We will discuss this seemingly non-specific binding later. However, we needed to determine the specificity of the localization of these agents when a drug is conjugated. We tested this using paclitaxel-FFRck-fVIIa [22] in multi-drug resistant KB-V1 SCC xenografts, to ascertain that the method will be useful for the
treatment of drug resistant cancers by conjugating a drug with potential activity in a particular drug resistant cancer. We needed to determine whether Cy5.5-labeled paclitaxel-FFRck-fVIIa [22] indeed accumulates specifically in the tumor target and spares other normal tissues/organs before undertaking anti-tumor angiogenesis therapy with paclitaxel-FFRck-fVIIa. Indeed, Cy5.5-labeled paclitaxel-FFRck-fVIIa appeared to specifically localize to KB-V1 SCC tumor xenografts and was only minimally localized to normal organs (Figure 5). The uptake of Cy5.5-labeled paclitaxel-FFRck-fVIIa was lower in the tumor xenograft of one mouse (number 4) than in the others. This tumor xenograft was found to be necrotic. These results suggest that Cy5.5-labeled paclitaxel-FFRck-fVIIa binds TF on the tumor endothelium and localizes in the tumor. Hence, it implies that we may be able to treat drug-resistant tumors by conjugating efficacious drugs that exert specific cytotoxicity in such tumors in the near future.

Figure 4. In vivo imaging of Cy5.5-FFRck-fVIIa and unconjugated Cy5.5 alone in mice carrying Mia PaCa-2 tumors that do not express TF. In each panel, the left animal was treated with Cy5.5 and the right animal with Cy5.5-FFRck-fVIIa. Cy5.5-FFRck-fVIIa bound TF in the tumor VECs of imaged tumors, but unconjugated Cy5.5 alone did not localize in the tumor on the right flank from day 1 through day 26 during the course of the experiment.
Discussion

In this study, we have developed a specific technique for imaging cancer in vivo using Cy5.5-labeled fVIIa, clotting-deficient FFRck-fVIIa, paclitaxel-FFRck-fVIIa, and anti-tissue factor (TF) antibody. These bind TF expressed on the luminal surface of tumor endothelial cells in addition to TF expressed in tumors [7–11]. It should be emphasized that Cy5.5-FFRck-fVIIa localized in the non-TF-expressing Mia PaCa-2 and TF-expressing ASPC-1 pancreatic tumor xenografts precisely in the same manner, indicating that the vasculature is capable of expressing TF even in the absence of tumoral TF expression. The common site of binding in both xenografts was TF-expressing endothelial cells within the tumor (Figure 1).

These findings are consistent with our previous demonstration that the same principle applies to the treatment of a human breast cancer model implanted subcutaneously and a lung metastasis model injected via tail vein with EF24-FFRck-fVIIa and paclitaxel-FFRck-fVIIa, respectively [18,23]. Targeting TF is important because it has been demonstrated to enhance tumor angiogenesis and tumor progression [4,6,12,13,15,26,28,29].

The significance of these findings is that this technology may be applicable for the detection of any tumor foci, for example, micro foci during pre- and post-surgery, and for the delivery of highly toxic drugs by conjugation with a specific carrier, for example, anti-TF antibody, TF8-5G9, which is an effective immediate anticoagulant in plasma. Binding of TF8-5G9 to TF-VIIa inhibits its catalytic function prior to dissociation of the TF-VIIa complex and does not trigger coagulation. This antibody should prevent coagulation in a similar manner to FFRck-fVIIa [30], and may be useful in treating GBM to disrupt the blood brain barrier and deliver potent drugs.

Since cancer patients are in the hypercoagulable state (Trousseau sign), it would seem preferable to use Cy5.5-labeled FFRck-fVIIa, a competitive inhibitor of fVIIa and/or Cy5.5-labeled anti-TF antibody, which inhibits the fVIIa/TF complex-induced coagulation cascade [20,30,31], as opposed to Cy5.5-fVIIa. This approach can be used to monitor the efficacy of EF24-FFRck-fVIIa [18,21] and paclitaxel-FFRck-fVIIa [22] treatment by using near-infrared fluorescence optical imaging of TF-expressing VECs and tumor cells in tumor xenografts including pancreatic cancer. Neither FVIIa nor FFRck-fVIIa binds normal tissues that lack TF expression [18,21,32].

It may be possible that there are non-specific binding properties of the Cy5.5 dye, as shown in Figures 3 and 4. Cy5.5 is lipophilic and its binding is pH-independent for pH ranges from 5 to 9. Since lipophilicity increases non-specific protein binding, Cy5.5 would be predicted to more strongly alter pharmacokinetics by nonspecific protein binding in vivo [33]. Our results indicate that the specific activity of the Cy5.5 conjugates lasts for approximately 1 week. The pharmacokinetic characteristics of Cy5.5-labeled-fVIIa and -humanized antibodies is associated with a prolonged circulation time and clearance times typically measured in several days to a week [32,34], whereas free Cy5.5 dye fluorescence in various organs was rapidly eliminated from 0.5 to 24 h after the i.v. injection [33]. It has been demonstrated that Cy5.5-labeled trastuzumab (anti-Her2 antibody) binds to HER-2 and is internalized. Subsequently it is catabolized and degraded within the endosome/lysosome. Likewise, the Cy5.5-FFRck-fVIIa may have been degraded and localized at non-specific sites due to non-specific cellular uptake of Cy5.5 [34].

We wondered why the lung uptake of paclitaxel-FFRck-fVIIa was higher than that in other normal tissues in KB-V1 SCC tumor xenografts (Figure 5). It has been demonstrated that TF is expressed on the bronchioles of the lungs, although TF is not expressed in the normal endothelium, and this may account for the higher binding in this organ [32].
To determine whether a drug-FFRck-fVIIa conjugate binds to TF with ability as FFRck-fVIIa in vivo, Cy5.5-labeled paclitaxel-FFRck-fVIIa was used. The Cy5.5-labeled paclitaxel-FFRck-fVIIa bound to TF on the tumor endothelium and was localized specifically to the tumor, with minimal off target binding. In Figure 5, the tumor of mouse 4 was necrotic, and contained fewer tumor blood vessels and tumor cells, hence there was less binding of Cy5.5-paclitaxel-FFRck-fVIIa to TF.

Together, these results imply that tumors may be detected in the early stage of development or during micro-metastasis using this highly specific method. Moreover, tumors that are drug-resistant to conventional therapies may be treated more effectively by conjugating efficacious drugs and targeting them to specific proteins to exert specific cytotoxicity.

In summary, this approach can be used to monitor the efficacy of EF24-FFRck-fVIIa [18,21] and paclitaxel-FFRck-fVIIa [22,23] and other chemotherapeutic treatments by using near-infrared fluorescence optical imaging of TF-expressing VECs and tumor cells in tumor xenografts of various cancers including pancreatic cancer, breast cancer and glioblastoma. Neither FVIIa nor FFRck-fVIIa bind normal tissues that lack VECs and tumor cells in tumor xenografts of various cancers.

**Conclusions**

To detect tumor localization and follow therapeutic response in vivo, Cy5.5-labeled FFRck-fVIIa, fVIIa and anti-TF antibody may be useful as we have demonstrated previously [18,21] and in this study. Our results suggest that paclitaxel-FFRck-fVIIa and EF24-FFRck-fVIIa conjugates will be specifically delivered to TF-expressing tumors and the tumor vasculature for targeted therapy.

**Declaration of interest**

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**References**