Near-Infrared Optical Imaging of Epidermal Growth Factor Receptor in Breast Cancer Xenografts

Shi Ke, Xiaoxia Wen, Michael Gurfinkel, Chusilp Charnsangavej, Sidney Wallace, Eva M. Sevick-Muraca, and Chun Li

ABSTRACT

The specificity of a novel epidermal growth factor (EGF)-Cy5.5 fluorescent optical probe in the detection of EGF receptor (EGFr) was assessed using continuous-wave fluorescence imaging accomplished via an intensified charge-coupled device (CCD) camera. Human mammary MDA-MB-468 (EGFr+) and MDA-MB-435 (EGFr-) cancer cells were incubated with Cy5.5, EGF-Cy5.5, or the anti-EGFr monoclonal antibody C225 or EGF followed by EGF-Cy5.5 and examined under a fluorescence microscope. In vivo imaging was performed on mice with s.c. MDA-MB-468 and MDA-MB-435 tumors. Images were obtained every 6 s for 20 min after i.v. injection of each agent and every 24 h after injection for up to 192 h. Additionally, mice with MDA-MB-468 tumors were injected i.v. with C225 24 h before injection of EGF-Cy5.5, EGF-Cy5.5, but not Cy5.5 or indocyanine green dye (ICG), bound to MDA-MB-468 cells. Binding of EGF-Cy5.5 was blocked by C225 and by EGF. In contrast, binding of EGF-Cy5.5 to MDA-MB-435 cells was not observed. Monitoring of the time-fluorescence intensity in mice confirmed that ICG and Cy5.5 had no favorable binding to tumor regardless of EGFr expression level. In contrast, EGF-Cy5.5 accumulated only in MDA-MB-468 tumors. Moreover, tumor uptake of EGF-Cy5.5 was blocked by C225. ICG and Cy5.5 fluorescence was completely absent from the tumor site, regardless of EGFr expression level, 24 h after injection. Little EGF-Cy5.5 fluorescence was detected in MDA-MB-435 tumors 24 h after injection. In MDA-MB-468 tumors, our data suggest that EGF-Cy5.5 may be used as a specific NIR contrast agent for noninvasive imaging of EGFr expression and monitoring of responses to molecularly targeted therapy.

INTRODUCTION

NIR optical imaging offers unique advantages for diagnostic imaging of solid tumors: it promises high sensitivity; it can be used to imaging a variety of molecular features because of its versatile fluorescent probe design; and it can provide dynamic, real-time in vivo images (1, 2). NIR light (700–1000 nm wavelength) can penetrate several centimeters into tissue, and thus NIR imaging offers a potentially safe, noninvasive means of characterizing diseased tissues. In most applications, NIR optical imaging is used in concert with molecularly targeted fluorescent contrast agents that not only provide enhanced contrast but also, more importantly, reveal specific molecular events associated with cancer formation and progression.

Prior work has established the use of antibody-mediated targeting of NIR excitable fluorescent contrast agents for the detection of tumors and the use of fluorophore-polymer conjugates that become fluorescent upon activation by matrix metalloproteases and intracellular enzymes present in tumors (3–6). However, in solid tumors, barriers to extravasation of large antibodies and polymeric complexes may impede transport of agents to cellular and intracellular molecular targets (7). An alternative approach has been demonstrated in vivo using small peptide-fluorophore conjugates for targeting of somatostatin (8, 9), bombesin, (10), and hydroxyapatite (for measurement of osteoblastic activity; Ref. 11).

EGFr is a transmembrane glycoprotein with an intracellular tyrosine kinase domain. EGFr and its ligands, including EGF, are frequently overexpressed in a variety of solid tumors, including cancers of the brain, colon, head and neck, lung, ovary, and pancreas (12, 13). Overexpression of EGFr is associated with increased metastatic potential and poor prognosis (14, 15). Given the importance of the EGFr pathways in tumor cell proliferation, many investigators have proposed anticancer approaches based on blocking the activity of EGFr. Monoclonal antibodies to the external domain of the EGFr have been developed to disrupt ligand binding to the receptor and subsequent signal transduction. Murine monoclonal antibody 225 and its chimeric counterpart C225 bind to EGFr, resulting in receptor down-regulation, blockage of EGFr-mediated tyrosine kinase activity, and inhibition of cellular proliferation (16). The effective use of new therapeutic agents that target EGFr will depend on the ability of physicians to detect and characterize EGFr-expressing lesions before and after the initiation of EGFr-directed treatments. EGFr scintigraphy, based on the binding, internalization, and retention of the radiolabeled, EGFr-targeting molecules in intracellular compartments, has been demonstrated with radiolabeled EGF and with radiolabeled monoclonal antibody directed against EGFr (17–19). These methods, however, require the use of harmful radioactive materials.

Here, we report on the specific imaging of EGFr in human breast tumor xenografts using NIR fluorophore-labeled EGF. We show that NIR images and intensity-time curves from dynamic NIR imaging might be used to characterize the presence of EGFr and to monitor therapies directed at EGFr.

MATERIALS AND METHODS

Materials. Recombinant human EGF (Mr 6,215) was purchased from Sigma Chemical Co. (St. Louis, MO). ICG was purchased from Akorn, Inc. (Buffalo Grove, IL). Monofunctional hydroxysuccinimide ester of Cy5.5 dye (Cy5.5-NHS) and PD-10 disposable columns were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Monoclonal antibody C225 was generously provided by ImClone Systems, Inc. (New York, NY). Mouse antirabbit IgG antibody was obtained from Pierce (Rockford, IL). Gel for SDS-PAGE was obtained from BioWhittaker Molecular Applications (Rockland, ME).

Synthesis and Characterization of EGF-Cy5.5. An aqueous solution of EGF (0.2 mg, 32 nmol) was mixed with four equivalents of Cy5.5-NHS and PD-10 disposable columns were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Monoclonal antibody C225 was generously provided by ImClone Systems, Inc. (New York, NY). Mouse antirabbit IgG antibody was obtained from Pierce (Rockford, IL). Gel for SDS-PAGE was obtained from BioWhittaker Molecular Applications (Rockland, ME).

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Requests for reprints: Chun Li, Department of Experimental Diagnostic Imaging, Box 59, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030. Phone: (713) 792-3182; Fax: (713) 794-5456; E-mail: c3li@dx.mdacc.tmc.edu.

1 The abbreviations used are: NIR, near-infrared; EGF, epidermal growth factor; EGFr, epidermal growth factor receptor; FPLC, fast protein liquid chromatography, ICG, indocyanine green dye; CCD, charge-coupled device.
1 ml/min. The ratio of Cy5.5 to EGF was estimated by measuring the absorbance of Cy5.5 and EGF in the EGF-Cy5.5 solution. Molar concentrations of Cy5.5 and EGF were calculated using extinction coefficients of 250,000 M⁻¹ cm⁻¹ at 678 nm for Cy5.5 dye and 18 M⁻¹ cm⁻¹ at 280 nm for EGF. Fluorescence properties were measured in PBS using a Fluorolog-3 spectrophotometer (Jobin Yvon, Inc., Edison, NJ) at an equivalent Cy5.5 concentration of 18 μM.

**Cell Lines.** Human breast adenocarcinoma cell lines MDA-MB-468 (EGF-repositive) and MDA-MB-435 (EGF-repositive) were obtained from American Type Culture Collection (Manassas, VA) and were maintained at 37°C in a humidified atmosphere containing 5% CO₂ in DMEM/nutrient mixture F-12 Ham culture medium (Chemical Co.) at 4°C for 18 h and 10% fetal bovine serum (Life Technologies, Inc., Grand island, NY).

**In Vitro Binding Studies.** Cells were seeded on coverslips in 24-well plates and incubated in DMEM/nutrient mixture F-12 Ham culture medium (0.5 ml/well) overnight. Cy5.5 (final concentration, 2.66 μM) or EGF-Cy5.5 (final concentration, 0.3 μM equivalent Cy5.5) was added into each well. For the blocking study, C225 (final concentration, 4.0 μM) or free EGF (final concentration, 10 μM) was added to the culture medium, and then Cy5.5-Cy5.5 was added 1 h later. After an incubation period of 1 h at 37°C, cells were washed twice with PBS and incubated in a solution of Sytox Green in 95% ethanol (1 μM; Molecular Probe, Eugene, OR) for 15 min to fix and stain cell nuclei. Cells were washed twice again with PBS, and the coverslips were mounted for microscopic examination using a DMR microscope (Leica Microsystems, Inc., Bannockburn, IL). The microscope was equipped with a 75-W Xenon lamp, 775 nm/845 nm and 480 nm/535 nm (Excitation/ emission) filters (Chroma Technology Corp., Brattleboro, VT), a Hamamatsu B/W chilled CCD camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan), and Image-Pro Plus 4.5.1 software (Media Cybernetics, Silver Spring, MD). In the microscopic images, Cy5.5 and Sytox Green were pseudocolored red and green (emission at 845 nm) and green (emission at 535 nm), respectively.

**Tumor Xenografts.** Four- to 6-week-old female athymic nude mice (18–22 g; Harlan Sprague Dawley, Inc., Indianapolis, IN) were housed five/cage and fed with sterilized pellet chow (Harlan Sprague Dawley, Inc.) and sterilized water. Animals were maintained in a pathogen-free mouse colony in the Department of Veterinary Medicine at The University of Texas M.D. Anderson Cancer Center. The facility is accredited by the American Association for Laboratory Animal Care, and all experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee. Tumor cells were harvested near confluence by incubation with 0.05% trypsin-EDTA. Cells were pelleted by centrifugation at 450 g for 5 min and resuspended in sterile PBS. Cells (2–3 × 10⁶/animal) were implanted s.c. into the chest walls of mice.

**In Vivo Optical Imaging System.** In vivo fluorescence imaging was accomplished by illuminating the animal with light from a laser diode (785 nm, 80 mW for ICG; 660 nm, 35 mW for Cy5.5 dyes) expanded to a circular area ~8 cm in diameter. The re-emitted fluorescent light was collected using an image intensifier (model FS9910C: ITT Night Vision, Roanoke, VA) lens coupled to a CCD camera (model CH350; Photometrics, Tucson, AZ). The imaging was designed so that the field of view could be varied from 3 × 3 cm to over 12 × 12 cm by varying the image distance of the 50-mm lens used to focus the image onto the photocathode of the intensifier. The lens was fitted with a holographic notch-plus filter (785-nm center wavelength for ICG and 660-nm center wavelength for Cy5.5 dyes; Kaiser Optical Systems, Inc., Ann Arbor, MI) and a bandpass filter (830-nm center wavelength for ICG, 710-nm center wavelength for Cy5.5 dyes) to reject back-scattered and reflected excitation photons. Image acquisition was accomplished using V + + software (Digital Optics, Auckland, New Zealand), and the obtained images were stored in uncompressed tagged image file format. Data processing and analysis was accomplished using Matlab software (The MathWorks, Inc., Natick, MA).

**Dynamic in Vivo Fluorescence Imaging.** Mice were anesthetized with an i.p. injection of 50 mg/kg pentobarbital, and a catheter was placed in the tail vein for i.v. injection of NIR dyes. A white-light image of the animal was obtained using a low-power lamp as a white-light source with holographic and interference filters removed. Fluorescence images were then acquired as a function of time after administration of 0.26 ml of ICG (1.0 nmol/mouse), Cy5.5 (2.9 nmol/mouse), or EGF-Cy5.5 (1.0 nmol equivalent Cy5.5/mouse). For the blocking study, 1.9 mg of C225 (12.7 nmol) was injected i.v. 2 h before the injection of EGF-Cy5.5. Images were obtained every 6 s for up to 20 min after injection of each contrast agent. Using the white-light image as a guide, two regions of interest of equivalent areas were designated, one corresponding to the tumor and the other corresponding to an area of normal tissue located symmetrically opposite the tumor region of interest. The mean fluorescence intensity from each region of interest was plotted as a function of time for the initial 20 min after injection of each contrast agent. Each mouse was imaged again 24 and 48 h after contrast injection. For the EGF-Cy5.5 conjugate in MDA-MB-468 mice, imaging was extended to 192 h after contrast. At the end of the imaging studies, mice were killed. Additional fluorescence images of the dissected organs were then obtained.

**Western Blot Analysis of EGFr Expression.** Tumors were collected and lysed using a Mammalian Cell Lysis Kit (Sigma Chemical Co.). Protein content in the lysate was quantitated using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). From each sample, an aliquot of tissue lysate containing 80 μg of protein was resolved by 10% SDS-PAGE. The proteins were electrophrotilled onto Immobilon-NC nitrocellulose membrane (Millipore, Bedford, MA). The membrane was blocked in 10% nonfat dry milk for 2 h at room temperature and incubated with monoclonal anti-EGFr antibody (Sigma Chemical Co.) at 4°C overnight. The membrane was then treated with horse-radish peroxidase-conjugated goat antimouse IgG + IgM secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at room temperature. Signal was detected using electrochemiluminescence Western blotting detection system (Amersham Biosciences).

**RESULTS**

Synthesis and Characterization of EGF-Cy5.5. The reaction scheme for synthesis of EGF-Cy5.5 is shown in Fig. 1. Each EGF molecule has one terminal amino group and two lysine side chain amino groups available for conjugation with Cy5.5. After reaction with Cy5.5-NHS, the product was purified using a PD-10 column. Size exclusion chromatography of purified EGF-Cy5.5 revealed successful removal of unreacted Cy5.5 (Fig. 2). The absorption and fluorescence emission characteristics of EGF-Cy5.5 conjugate were similar to those of free Cy5.5, as apparent from the spectra measured in PBS (data not shown), suggesting that the fluorescence property of Cy5.5 was not affected by conjugation to EGF. The molar ratio of Cy5.5 to EGF was estimated to be 0.5. The yield of EGF was typically 80–86%.

**In Vivo Binding Studies.** Fig. 3 compares fluorescence images of MDA-MB-468 and MDA-MB-435 cells incubated with Cy5.5 or EGF-Cy5.5. Negligible signals were detected in both cell lines when cells were incubated with Cy5.5 dye (Fig. 3, A and B). In contrast, EGF-Cy5.5 bound to MDA-MB-468 cells but not to MDA-MB-435 cells (Fig. 3, C and D). Furthermore, binding of EGF-Cy5.5 to...
MDA-MB-468 cells was completely blocked by anti-EGFr antibody C225 (13-fold excess; Fig. 3E) and by EGF ligand (33-fold excess; Fig. 3F).

**In Vivo Fluorescence Imaging with EGF-Cy5.5.** To confirm the expression of EGFr in tumors grown in nude mice, Western blot analysis of excised tumors was performed. Consistent with data previously reported in the literature (19), MDA-MB-468 tumors had high levels of EGFr expression, whereas MDA-MB-435 tumors did not express EGFr (data not shown).

Fig. 4A compares typical NIR fluorescence images of mice bearing EGFr-positive MDA-MB-468 tumors and EGFr-negative MDA-MB-435 tumors immediately and at 24 and 48 h after i.v. injection of EGF-Cy5.5. Fluorescence signal was clearly visualized in MDA-MB-468 tumors but not in MDA-MB-435 tumors. Significant fluorescence was still detectable in MDA-MB-468 tumors at 96 h after contrast injection (data not shown). The signal intensity fell back to the background level by 192 h after contrast injection (data not shown). Dissection analysis revealed that the optical activities observed in the chest and pelvic areas of the mice mainly represented contributions from the gastrointestinal tract and from the liver (Fig. 4B). Images of mice without contrast agent injected showed autofluorescence in the stomach and the gastrointestinal tract (data not shown). Images of mouse chow and its aqueous extract also showed strong fluorescence signal (data not shown).

Fig. 5 shows NIR fluorescence images of mice with MDA-MB-468 tumors injected with either EGF-Cy5.5 (Fig. 5A) or C225 followed by injection of EGF-Cy5.5 24 h later (Fig. 5C). Results of quantitative measurement of the fluorescence intensity 24 h after contrast injection at each pixel on lines through the tumor region and region of greatest intensity are also plotted (Fig. 5, B and D). In mice injected with EGF-Cy5.5, the intensity in the tumor region was significantly higher than the background. However, when mice were pretreated with C225, tumors were not seen (Fig. 5C), and the intensity on the line through the tumor region was reduced to close to the background level (Fig. 5D). Preinjection with a nonspecific IgG antibody did not affect the uptake of EGF-Cy5.5 in MDA-MB-468 tumors (data not shown).

**Comparison of Kinetics of NIR Dye Uptake in Tumors and Normal Tissue.** The fluorescence intensities in the tumor and the normal tissue were measured dynamically during the initial 20 min after injection of ICG, Cy5.5, or EGF-Cy5.5 in tumor-bearing mice. Fig. 6 illustrates the mean fluorescence intensity as a function of time for the regions of interest corresponding to tumor and normal tissues. Little difference was noted between the rate of uptake in the tumor region and the rate of uptake in the normal tissue region after injection of Cy5.5 or ICG (Fig. 6, A, C, and D). In mice with MDA-MB-468
tumors, the rate of uptake of EGF-Cy5.5 was significantly faster in tumor than in the normal tissue (Fig. 6F). On the other hand, in mice with MDA-MB-435 tumors, there was no difference in the rate of uptake of EGF-Cy5.5 between tumors and normal tissue (Fig. 6B). In mice with MDA-MB-468 tumors, pretreatment with C225 resulted in suppression of EGF-Cy5.5 uptake in the tumors (Fig. 6E).

DISCUSSION

NIR optical imaging is a valuable tool for studying the status of receptor expression noninvasively in small animals, provided that an appropriate NIR probe targeting specific receptors is available. In this article, we describe the use of EGF-Cy5.5 conjugate to image EGFr in...
human breast tumor xenografts. The conjugate was conveniently synthesized from EGF and NHS ester of Cy5.5 (Fig. 1). Conjugation of Cy5.5 to EGF did not affect its optical properties: EGF-Cy5.5 and Cy5.5 had similar absorbance and emission spectra. Unreacted Cy5.5 was simply removed using a gel permeation PD-10 column (Fig. 2).

Specific binding of EGF-Cy5.5 to tumor cells expressing EGFr was established by comparing the binding of EGF-Cy5.5 to EGFr-overexpressing MDA-MB-468 tumor cells and to EGFr-negative MDA-MB-435 tumor cells. Although no fluorescence signal was detected in MDA-MB-435 cells incubated with EGF-Cy5.5, fluorescence signal was observed in MDA-MB-468 cells incubated with EGF-Cy5.5 (Fig. 3). The possibility of nonspecific binding to free Cy5.5 was excluded by our finding that no fluorescence signal was detected in cells incubated with Cy5.5 (Fig. 3, A and B). Moreover, when MDA-MB-468 cells were pretreated with either the anti-EGFr antibody C225 or EGF itself, binding of EGF-Cy5.5 to the cells was blocked (Fig. 3, E and F), indicating that binding of EGF-Cy5.5 to MDA-MB-468 cells was mediated through EGFr.

Consistent with our in vitro findings, specific targeting of EGF-Cy5.5 in vivo to EGFr was demonstrated by comparing the NIR images of mice bearing EGFr-expressing MDA-MB-468 tumors with those of mice bearing EGFr-negative MDA-MB-435 tumors. Although no fluorescence signal was visualized in MDA-MB-435 tumors, accumulation of EGF-Cy5.5 in MDA-MB-468 tumors was clearly seen (Fig. 4B). Furthermore, the uptake of EGF-Cy5.5 in MDA-MB-468 tumors was blocked by the anti-EGFr antibody C225, a result confirmed by quantitative analysis (Fig. 5). Therefore, both in vitro and in vivo studies support that EGF-Cy5.5 specifically targets EGFr.

It is interesting that the fluorescence signals detected in the chest and the pelvic areas were primarily from the liver and the gut (Fig. 4C). Liver is known to express high levels of EGFr (19, 20), which would explain the high level of liver uptake of EGF-Cy5.5. We speculate that the fluorescence signal from the gastrointestinal tract may have resulted from the mouse diet. In fact, both solid mouse chow and its aqueous extract showed strong NIR fluorescence signal. In the future, it may be necessary to feed mice with a diet depleted of fluorescent substances to reduce background signal and improve imaging quality. Alternatively, one could use an NIR dye with a longer emission wave number and an appropriate filter set to reduce the interference from the background. Both approaches are currently being explored in our laboratories.

A comparison of the pharmacokinetics of targeted imaging probes in tumor and in normal tissues can help gain insight into the specificity of the probe used. The kinetics of EGF-Cy5.5 uptake in tumors and in normal tissues (muscle) as seen in serial NIR images were compared (Fig. 6). Dynamic measurement of fluorescence intensities in the first 20 min after contrast injection clearly demonstrated differences in the rates of uptake of ICG and Cy5.5, on the other hand.
hand, did not differ significantly between tumors and muscle in mice with MDA-MB-468 tumors or mice with MDA-MB-435 tumors (Fig. 6, hand, did not differ significantly between tumors and muscle in mice with MDA-MB-468 tumors). Pretreatment with C225 also resulted in a decreased rate of uptake of EGF-Cy5.5 and a decreased gap between the rates of EGF-Cy5.5 uptake in tumors and in muscle in mice with MDA-MB-468 tumors (Fig. 6E). These data again support the notion that EGF-Cy5.5 uptake was taken up by MDA-MB-468 tumor through a receptor-mediated process. Significantly, this conclusion was drawn independently on the basis of a 20-min dynamic imaging session; there was no need to wait for hours for the contrast agent to clear from the blood pool.

In conclusion, our study underscores the potential benefits of NIR optical imaging in small animal molecular imaging, specifically the ability to conduct fast fluorescence measurements at a low fluorophore dose (1 nmol/mouse in this work). Such features may provide a noninvasive means for the detection and characterization of EGFr-expressing lesions without the harmful effect of radiation rays (i.e., in nuclear imaging), enhancing our ability to assess the effects of therapeutic interventions that target EGFr.

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