Spectral Fluorescence Molecular Imaging of Lung Metastases Targeting HER2/neu

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Abstract Purpose: Surgical resection of pulmonary metastases is now a clinically accepted cancer therapy but its success depends on the accurate localization and removal of all tumor foci. To enhance the detection of pulmonary metastases during surgery, we developed an i.v. administered optical probe that uses a monoclonal antibody, Herceptin (trastuzumab), conjugated to a fluorophore, rhodamine green (RhodG), to specifically detect human epidermal growth factor receptor type 2 (HER2/neu) – expressing pulmonary lesions in an animal model of lung metastases.

Experimental Design: Pulmonary metastases were induced by i.v. injection of gene-transfected murine embryonic fibroblasts (3T3) cells in a murine model to produce a mixed population of HER2+ and HER2− tumors. To image these tumors, an anti-HER2 (Herceptin) or a control (HUT) complementarity-determining region – grafted antibody was conjugated to RhodG and injected i.v. into mice. Spectral fluorescence imaging was done after thoracotomy and images were correlated with gross and microscopic pathology to assess sensitivity and specificity.

Results: HER2+ tumors injected with Herceptin-RhodG were more fluorescent than either HER2− tumors or HER2+ tumors injected with HUT-RhodG at all time points. The maximal fluorescence signal in HER2+ tumors injected with Herceptin-RhodG was observed at 1 day post-injection. The tumors fluoresced primarily at the rim and not their center, reflecting the binding-site barrier that is commonly seen with high-affinity antibodies.

Conclusion: A HER2-targeted optical imaging probe shows the ability to specifically enhance HER2+ pulmonary metastases but not HER2− pulmonary metastases. The high sensitivity and specificity of this probe is encouraging for the development of antigen-targeted optical probes to assist in the resection of pulmonary metastases.

Pulmonary metastases are a major cause of morbidity and mortality. In selected patients, surgical metastasectomy has become an accepted treatment and is reported to prolong survival in many cancers (1, 2). Although complete resections are thought to occur in the majority of patients, relapse rates can be as high as 70% to 80% and recurrence in the lung can be as high as 30% to 40% (3), indicating that small foci of metastatic disease may elude resection. Methods to enhance intraoperative identification of tiny pulmonary metastases could improve the completeness of surgical resection and, hence, patient survival (4, 5). Ultimately, such methods could aid in patient selection for metastasectomy. Optical fluorescent probes are attractive for intraoperative enhancement because they allow real-time, high-resolution imaging without the need for ionizing radiation or complex equipment. Untargeted optical probes, however, enhance both the tumor and background, making detection difficult. Molecularly targeted optical probes, however, allow the specific uptake of the fluorophore by the tumor whereas unbound conjugate is eliminated systemically, resulting in high target signal-to-background ratios, which, in turn, permit the detection of submillimeter clusters of cancer cells.

To test targeted optical fluorescent agents, three critical components of the experiment must be considered: the animal model, the targeting ligand, and the fluorophore. A major challenge to this type of research is the development of animal models of metastatic disease that not only replicate human disease but also allow the determination of sensitivity and specificity of the targeted optical agent. Evaluating two populations of mice with different cell lines, one targeted and one control, is adequate for semiquantitative side-by-side comparisons. However, evaluating two separate populations of mice is not appropriate for analyzing sensitivity/specitivity because it is difficult to maintain identical experimental conditions and imaging variables in different subjects. Animal models in which both targeted and control tumors are expressed...
in the same animal permit imaging under the same experimental and imaging conditions. Furthermore, by transfecting control tumors with a fluorescent protein like red fluorescent protein (RFP) or green fluorescent protein, which constitutively fluoresce, it is possible to determine the sensitivity and specificity of the agent in the same animal by comparing in situ imaging with ex vivo imaging and histopathologic correlation. For the model to be realistic, it is also critical that the target and control tumors differ primarily in their ability to express the target but not in other biological properties.

Although many potential targeting vectors exist, monoclonal antibodies have several advantages. A number of humanized monoclonal antibodies (e.g., trastuzumab, bevacizumab, and cetuximab) have already received clinical approval as human cancer therapies and, therefore, are potentially more readily translated into human clinical trials. Trastuzumab or Herceptin has a high binding affinity for the cell-surface marker human epidermal growth factor receptor type 2 (HER2/neu; erbB2/p185; ref. 6) and, therefore, could be potentially useful in HER2-expressing pulmonary metastases (7, 8). A potential limitation of high-affinity antibodies is that they preferentially bind to the outer tumor layers and only slowly migrate into the center of the tumor, a limitation known as the “binding site barrier” (9). Although the binding site barrier is one of the major obstacles for radioimmunotherapy with radioisotope-armed antibodies, the extent to which this might limit the diagnostic capabilities of antibody-labeled optical probes is unknown.

Finally, a third challenge is determining the best fluorophore to use in the targeted imaging conjugate. A growing number of high-efficiency organic fluorophores have been developed over the past 10 years. Whereas their fluorescence can be readily tested in vitro, their behavior in situ, particularly after they have been internalized within targeted cells, has not been thoroughly explored. In this study, we describe a novel animal model of pulmonary metastases wherein two separate tumor types, one that is HER2+ but is not normally fluorescent and one that is HER2− but is constitutively fluorescent, is used to test the sensitivity/specificity of a targeted optical agent. Further, we describe the synthesis and testing of the monoclonal antibody-fluorophore conjugate Herceptin-rhodamine green (Herceptin-RhodG) after testing other organic green fluorophores. Using this system, we determine the sensitivity and specificity of Herceptin-RhodG for the detection of HER2+ pulmonary metastases compared with a control antibody conjugate (HUT-RhodG), which does not target HER2+ cells.

### Materials and Methods

**Synthesis of Herceptin-conjugated green fluorescent dyes.** Herceptin, a Food and Drug Administration–approved humanized anti-human epidermal growth factor receptor type 2 (HER2) antibody, generically known as trastuzumab, which has a complementarity-determining region (CDR) against HER2 grafted on a human immunoglobulin G1 framework (6), was purchased from Genentech. As a control antibody, humanized anti-Tac (Zenapax; HUT), which is a Food and Drug Administration–approved humanized anti-human interleukin-2 receptor α-subunit antibody with a CDR against interleukin-2 receptor α-subunit grafted on a human immunoglobulin G1 framework (10), was obtained as a generous gift from Dr. Thomas Waldman (Metabolism Branch, National Cancer Institute/NIH, Bethesda, MD). HUT is a good control antibody for Herceptin because it is isootype matched and shows >98% protein sequence homology with Herceptin.

Amido-reactive fluorescein (FITC), BODIPY-FL (BODIPY), Oregon green (Oreg), and RhodG were purchased from Molecular Probes, Inc. At room temperature, 500 μg (3.3 nmol) of Herceptin or HUT in Na3HPO4 were incubated with 10 to 100 nmol (1-10 μL/10 nmol/L) of isothiocyanato-benzyl-fluorescein (FITC), BODIPY, Oreg, or RhodG-succinimidyl ester, respectively, in DMSO at pH 8.5 for 15 min. The mixture was purified with a Sephadex G50 column (PD-10; GE Healthcare). Herceptin-conjugated fluorescein, BODIPY, Oreg, and RhodG samples (Herceptin-FITC, Herceptin-BODIPY, Herceptin-Oreg, and Herceptin-RhodG, respectively) were kept at 4°C in the refrigerator as stock solutions.

The protein concentration of Herceptin-FITC, Herceptin-BODIPY, Herceptin-Oreg, and Herceptin-RhodG samples was determined with Coomassie Plus protein assay kit (Pierce Biotechnology) by measuring the absorption at 595 nm with a UV-Vis system (8453 Value UV-Vis system, Agilent Technologies) using standard solutions of known concentrations of Herceptin (100, 200, and 400 μg/mL). Then, the concentration of fluorescein, BODIPY, Oreg, and RhodG was measured by the absorption at 497, 508, 500, and 503 nm, respectively, with the UV-Vis system to confirm the number of fluorophore molecules conjugated with each Herceptin molecule. By changing the concentration of Herceptin solution, the number of fluorophore molecules per Herceptin was adjusted to ~0.8.

In quality control, the Herceptin conjugates were analyzed with SDS-PAGE (4-20% Tris-glycine SDS gel; Invitrogen) under reducing conditions. Fluorescence associated with antibody heavy and light chains as well as small molecules using a high resolution imaging system was measured as a percent of total fluorescence (FLA-5100, Fujifilm Medical Systems USA, Inc.). Almost all the fluorescence from antibody conjugates (95 ± 4%) was associated with binding to heavy-chain and light-chain bands.

**Tumor cells.** NIH 3T3 transfected with the HER2 gene (3T3/HER2), which overexpressed HER2 receptors (~2 × 10^6 per cell), and Balb/3T3/HER2− cell lines (American Type Culture Collection; refs. 11, 12) were used for in vitro fluorescence microscopy, flow cytometry, and in situ postmortem optical imaging for pulmonary metastasis. The cell lines were grown in RPMI 1640 (Life Technologies, Inc.) containing 10% fetal bovine serum (Life Technologies), 0.03% l-glutamine at 37°C, 100 units/mL penicillin, and 100 μg/mL streptomycin in 5% CO2.

**Rhod2** transfection of Balb/3T3 cells. The RFP (DsRed2)–expressing plasmid was purchased from Clontech Laboratories, Inc. The plasmid was transfected into the Balb/3T3 cells to validate the results with the targeted fluorophores (see below). The transfection of RFP was done with the electroporation method using Gene Pulser II (Bio-Rad Laboratories). Briefly, 3 μg of DsRed2–express plasmid were mixed with 2 million Balb/3T3/HER2− cells in 400 μL of the cell culture medium (RPMI 1640 with 10% FCS). Then the cell suspension was placed in a pulse cuvette (Bio-Rad Laboratories) and 250-V pulses were delivered after 950 cycles. Then, the Balb/3T3/HER2−/−RFP+ cell was cloned with the limiting dilution culture method (13).

**Fluorescence microscopy.** 3T3/HER2+ cells (1 × 10^6) were plated on a cover glass bottom culture well and incubated for 16 h. Herceptin-FITC, Herceptin-BODIPY, Herceptin-Oreg, or Herceptin-RhodG was added to the medium (30 μg/mL) and the cells were incubated for 1 h. Cells were washed once with PBS and fluorescence microscopy was done immediately and 8 h after washing using an Olympus BX61 microscope (Olympus America, Inc.) equipped with the following filters: excitation wavelength, 470 to 490 nm; emission wavelength, 515-nm long pass. Transmitted light differential interference contrast images were also acquired.

**Flow cytometry.** One-color flow cytometry was done for the assessment of the specificity of Herceptin-RhodG against 3T3/HER2+ cells and a comparison was made of the fluorescence of the four green
dyes after cell labeling by Herceptin-FITC, Herceptin-BODIPY, Herceptin-OreG, and Herceptin-RhodG. 3T3/HER2+ cells (1 × 10⁶) were plated on a 12-chamber well and incubated for 16 h. Herceptin-FITC, Herceptin-BODIPY, Herceptin-OreG, or Herceptin-RhodG was added to the medium (1 μg/mL) and the cells were incubated for 1, 2, 4, and 8 h. One hour after their incubation with each of the four dyes, the cells were washed twice with PBS, trypsinized, and flow cytometry was done immediately 1, 2, 4, and 8 h after washing. A 488-nm argon-ion laser was used for excitation. Signals from cells were collected using a 350/30-nm band-pass filter. Cells were analyzed in a FACScan flow-meter (Becton Dickinson) and all data were analyzed using CellQuest software (Becton Dickinson). The light emission from each fluorophore was measured as the mean fluorescence index (MFI) and the MFI fading rate was determined by the washout slope of MFI as a function of incubation time.

Comparison of signal intensity of fluorophores conjugated with Herceptin. To compare the fluorescence of the four fluorescent Herceptin conjugates, fluorescence intensity and emission spectra of Herceptin-BODIPY, Herceptin-FITC, Herceptin-OreG, and Herceptin-RhodG were measured in arbitrary units with the Maestro In-Vivo Imaging System (CRI, Inc.). Ten micrograms of Herceptin-BODIPY, Herceptin-FITC, Herceptin-OreG, and Herceptin-RhodG in phosphate buffers with different pH values in 390-μL phosphate buffers (pH 3.3, 5.2, 6.4, and 7.4) were placed in a nonfluorescent 96-well plate and spectral fluorescence imaging was done. A band-pass filter from 445 to 490 nm and a long-pass filter over 515 nm were used for emission and excitation light, respectively. The tunable filter was automatically stepped in 10-nm increments from 500 to 800 nm whereas the camera captured images at each wavelength interval with constant exposure time. Spectral unmixing algorithms were applied to create the image of each of the green dyes. A region of interest was drawn at the center of each well to determine the fluorescence intensity as well as the emission spectrum of each dye conjugated with Herceptin using ImageJ software. For the assessment of signal intensity under acidic conditions, the regression line of fluorescence intensity in each dye was calculated as a function of pH value. The measurement was repeated four times in each well and fluorescence intensity was expressed as mean arbitrary units ± SD. Statistical analysis was done using a paired Student’s t test for each combination of two samples.

Tumor model. All procedures were carried out in compliance with the Guide for the Care and Use of Laboratory Animal Resources (1996), National Research Council, and approved by the local Animal Care and Use Committee. The metastatic lung tumors were established by i.v. injection of 2 × 10⁶ cells suspended in 200 μL of PBS in female nude mice (National Cancer Institute Animal Production Facility, Frederick, MD). Side-by-side comparative experiments with lung metastatic tumor-bearing mice to evaluate the sensitivity, the specific accumulation, and the semiquantitative analysis of Herceptin-RhodG in the 3T3/HER2+ tumors were done at 14 days postinjection of the nontransfected Balb/3T3/HER2− cells or at 21 days postinjection of 3T3/HER2+ cells. Two-color experiments with two injected cell lines of lung metastases in mice (RFP-transfected Balb/3T3/HER2−/RFP+ and 3T3/HER2+) were conducted to evaluate the sensitivity and specificity of detecting HER2+ expressing tumors. Tumor detection were done at 12 days (Balb/3T3/HER2−/RFP+) and 19 days (3T3/HER2+ postinjection of cells).

In situ postmortem spectral fluorescence imaging. To evaluate the sensitivity and specific accumulation and to carry out a semiquantitative analysis, side-by-side comparative experiments were done. Fifty micrograms of Herceptin-RhodG or HuT-RhodG as a control antibody were diluted in 200 μL of PBS and injected into the tail vein of mice with lung metastatic tumors. After 1, 2, 4, or 7 days postinjection of agent, each mouse was sacrificed with carbon dioxide. Immediately after sacrifice, the pleural cavity was exposed, and two mice, each bearing the same tumor but different fluorophore conjugates or bearing different (3T3/HER2+ or Balb/3T3/HER2−) tumors and the same fluorophore conjugate, were placed side by side on a nonfluorescent plate to compare the fluorescence intensity of tumors simultaneously. Spectral fluorescence images were obtained using the Maestro In-Vivo Imaging System. In situ postmortem lung images as well as close-up ex vivo images were obtained. A band-pass filter from 445 to 490 nm and a long-pass filter over 515 nm were used for emission and excitation light, respectively. The tunable filter was automatically stepped in 10-nm increments from 500 to 810 nm whereas the camera captured images at each wavelength interval with constant exposure. The spectral fluorescence images consisting of lung and muscle autofluorescence spectra and RhodG spectra were obtained and then were unmixed based on their spectral patterns using commercial software (Nuance ver. 1.4, Maestro software, CRI). The experiment was repeated four to five times for each pair of animals. All investigations took part in a region of the Maestro camera field of view that was previously documented to have 80% homogeneity, so that changes in light emission related to inhomogeneous illumination could account for no more than 20% of the variation in signal.

To evaluate the sensitivity and specificity of HER2+ tumor detection, two-color experiments were done in which the same animal had both HER2+ and HER2− tumors. Fifty micrograms of Herceptin-RhodG were diluted in 200-μL PBS and injected into the tail vein of these mice. One or 2 days after injection of the agent, each mouse was sacrificed with carbon dioxide. Immediately after sacrifice, the pleural cavity was exposed and the mouse was placed on a nonfluorescent plate. Then, spectral fluorescence images were obtained using the Maestro In-Vivo Imaging System. In situ postmortem lung images as well as close-up ex vivo images were obtained with the same method as described above. The spectral fluorescence images, consisting of lung autofluorescence spectra and spectra from the RhodG spectra and the RFP spectra, were obtained and then unmixed based on their spectral patterns using Nuance ver. 1.4 and a composite image consisting of RhodG (green) and RFP (red) signals and lung autofluorescence (white).

To determine the sensitivity and specificity of Herceptin-RhodG for detecting HER2+ tumors on the spectral fluorescence imaging, four mouse lungs at 1 day postinjection of Herceptin-RhodG were examined. Detection of green (RhodG-positive) tumors and red (RFP-positive) tumors was subjectively and independently done using magnified fluorescent composite images and stereoscopic microscope images on a 17-in. flat screen monitor (NEC) by two certified radiologists (Y.K. and H.K.) with experience in mouse imaging.

Two-color fluorescence microscopic imaging. Immediately after finishing the fluorescent imaging study, the dissected lung specimens were frozen in Tissue-Tek (Sakura) and stored at −80°C. Frozen samples were sectioned with a cryostat microtome (CM1800, Leica) at a thickness of 30 μm. Slides were analyzed under an Olympus BX61 microscope (Olympus America) equipped with the following filters: FITC cube set for RhodG; excitation wavelength 470 to 490 nm, emission wavelength 515-nm long pass, TexRed cube set for RFP; excitation wavelength 530 to 570 nm, emission wavelength 590 nm long pass. Transmitted light differential interference contrast images were also acquired. Two-color composite images of fluorescence were generated using the standard software on the fluorescence microscope (MicroSuite Five Olympus America).

To determine the sensitivity and specificity of HER2+ tumors with Herceptin-RhodG by fluorescence microscopic imaging, the same four mouse lungs at 1 day postinjection of Herceptin-RhodG were examined microscopically. Detection of green (RhodG-positive) tumors and red (RFP-positive) tumors was subjectively and independently done using magnified 12 fluorescence microscopic composite images (~40) and corresponding differential interference contrast images up to the full screen on a 17-in. flat screen monitor by two certified radiologists (Y.K. and H.K.).
Results

In vitro fluorescence under neutral and acidic conditions. To select the optimum green fluorescent dye for animal experiments, four green fluorescent dyes, BODIPY, fluorescein (FITC), OreG, and RhodG, were conjugated to Herceptin to compare their fluorescence intensities and emission spectra in neutral and acidic conditions. The fluorescence of these probes at low pH conditions is important because it is expected that the fluorophore conjugates will be internalized into target cells and accumulate in the endosomes or lysosomes where they will be exposed to acidic conditions. The comparison was done using the same dose of Herceptin (10 μg) and the same number of fluorophores conjugated per Herceptin (~0.8). Spectral analysis yielded a peak emission wavelength of 550 ± 10 nm for all four conjugates (Fig. 1A). The fluorescence intensities of Herceptin-BODIPY, Herceptin-FITC, Herceptin-OreG, and Herceptin-RhodG in 390-μL PBS (pH 7.4) were 4,398 ± 400, 7,380 ± 860, 8,371 ± 710, and 5,867 ± 115 arbitrary units, respectively (n = 4; P < 0.01 for all combinations; Fig. 1B). When these four dyes (10 μg each) were placed in an acidic environment (390-μL phosphate buffer, pH 3.3), the fluorescence intensities of Herceptin-BODIPY, Herceptin-FITC, Herceptin-OreG, and Herceptin-RhodG were 3,469 ± 115, 1,437 ± 27, 2,646 ± 413, and 5,295 ± 324 arbitrary units, respectively (n = 4; P < 0.01 for all combinations; Fig. 1B). The slopes of regression lines calculated as a function of pH values were 196, 1,437, 1,404, and 123 for Herceptin-BODIPY, Herceptin-FITC, Herceptin-OreG, and Herceptin-RhodG, respectively. These results indicate that the fluorescence intensities of Herceptin-FITC and Herceptin-OreG decrease under acidic conditions, whereas the fluorescence intensities of Herceptin-BODIPY and Herceptin-RhodG are maintained or increase under acidic conditions. Herceptin-BODIPY and Herceptin-RhodG were thus considered the most promising candidates for in vivo imaging.

Fluorescence in cell culture. 3T3/HER2+ cancer cells were incubated with each of the four green fluorophore conjugates to determine their intracellular fluorescence. Serial flow cytometry was done after 1, 2, 4, and 8 h of incubation with each of the fluorophore conjugates. The MFI values of Herceptin-BODIPY, Herceptin-FITC, Herceptin-OreG, and Herceptin-RhodG at 1 h postincubation were 87, 262, 805, and 453 absorbance units, respectively, with Herceptin-OreG the highest, followed by Herceptin-RhodG, Herceptin-FITC, and Herceptin-BODIPY (Fig. 2A). During the incubation period of 8 h, MFI values of Herceptin-BODIPY and Herceptin-OreG changed little, whereas the MFI value of Herceptin-FITC decreased and that of Herceptin-RhodG increased (Fig. 2B). To investigate the intracellular fluorescence dynamics, serial flow cytometry was done after washing each of the fluorophore conjugates. Herceptin-BODIPY, Herceptin-FITC, and Herceptin-OreG showed a similar decline in fluorescence signal over 8 h after washing (Fig. 2C). The relationship between fluorescence (log value of MFI) as a function of postincubation time was expressed as the slope of the regression line (log MFI versus time) for Herceptin-BODIPY, Herceptin-FITC, Herceptin-OreG, and Herceptin-RhodG and was −0.0285/h, −0.0342/h, −0.0219/h, and −0.0003/h, respectively. These results indicate that the MFI of Herceptin-FITC decreases most rapidly (highest negative slope) whereas Herceptin-RhodG maintains strong fluorescence even after long incubation times.
(lowest negative slope). Thus, Herceptin-RhodG was selected as the optimized targeted fluorophore.

**Fluorescence microscopy.** To investigate the intracellular location of Herceptin-conjugated fluorophores, *in vitro* fluorescence microscopy and differential interference contrast images were obtained on 3T3/HER2+ cells immediately and 8 h after washing each of the four fluorophore conjugates. Cells were incubated with each of the four fluorophore conjugates for 1 h before washing. In all four fluorophore conjugates, fluorescence microscopy showed high signal on the cell surface and in the cytoplasm immediately after washing (Fig. 3). At 1 h, all the fluorophore conjugates behaved similarly. However, 8 h after incubation, the fluorescence of Herceptin-BODIPY, Herceptin-FITC, and Herceptin-OreG decreased whereas the intracellular fluorescent dots produced by Herceptin-RhodG became larger and brighter than the other three fluorophores (Fig. 3). These results indicate that all four dyes are internalized into 3T3/HER2+ cancer cells, but Herceptin-RhodG maintains the highest fluorescence over time.

**Herceptin-RhodG specifically accumulates in HER2+ cells.** To confirm that Herceptin-RhodG specifically binds to HER2+ cells, flow cytometry of 3T3/HER2+ and Balb/3T3/HER2− cells was done 3 h after incubation with Herceptin-RhodG. The percent of labeled cells was 2% for Balb/3T3/HER− and 100% for 3T3/HER2+ cells, indicating strong specificity of Herceptin-RhodG (Fig. 4A and B). Fluorescence microscopy confirmed that Herceptin-RhodG was bound and internalized into 3T3/HER2+ cells whereas HUT-RhodG did not bind or internalize into 3T3/HER+ cells. Moreover, Herceptin-RhodG did not bind to Balb/3T3/HER− cells (Fig. 4C).

**Herceptin-RhodG specifically visualizes HER2+ metastatic tumors in situ.** Serial images of pairs of mice with 3T3/HER2+ metastatic tumors, one injected with Herceptin-RhodG and the other with HUT-RhodG, showed specific accumulation of Herceptin-RhodG in the tumor at all time points after injection (Fig. 5A and B). *In situ* postmortem and *ex vivo* spectrally unmixed and composite images showed that the fluorescence signal produced by RhodG originated only from the tumor sites (Fig. 5B and C). In addition, serial images of pairs of mice with 3T3/HER2+ and Balb/3T3/HER2− tumors showed specific accumulation of Herceptin-RhodG into the 3T3/HER2+ metastatic nodules at all time points after injection whereas Balb/3T3/HER2− tumors showed minimal or no accumulation (Fig. 5A and B). In all *in situ* postmortem and *ex vivo* images of the lung (Fig. 5A and D) as well as fluorescence microscopic images (Fig. 5D) on resected specimens, Herceptin-RhodG accumulated at the rim of 3T3/HER2+ metastatic nodules at 1 day postinjection but was distributed more homogeneously throughout the nodules at later time points. These findings are consistent with the “binding site barrier effect” reported previously for high-affinity macromolecular targeting molecules such as monoclonal antibodies. Interestingly, the maximal fluorescence signal of Herceptin-RhodG was seen at 1 day corresponding to maximal rim enhancement (Fig. 5A and D).

**Sensitivity and specificity of Herceptin-RhodG for 3T3/HER2+ tumors.** In *in situ* postmortem and *ex vivo* spectral imaging (Fig. 6A) was able to distinguish between 3T3/HER2+ (green fluorescence spectra) and Balb/3T3/HER2−/RFP+ (red fluorescence spectra) nearly without any coincidence (yellow) of both colors. In *ex vivo* lung images shown in Fig. 6A, true positive...
3T3/HER2+ lesions were defined as having green fluorescence and true positive Balb/3T3/HER2+/RFP+ tumors were defined as having red fluorescence due to the expression of RFP in these cells. Among 490 3T3/HER2+ tumors, Herceptin-RhodG detected all 490 for a sensitivity of 100%. Among the 732 resected Balb/3T3/HER2+/RFP+ tumors, Herceptin-RhodG detected 27 (false positives) for a specificity of 96.3%. All tumors detected with a stereoscopic microscope under the white light showed either green or red fluorescence (no false negatives). Fluorescence microscopic images of 20 randomly selected lung specimens, which were harvested after \textit{ex vivo} imaging, validated that Herceptin-RhodG accumulated only in 3T3/HER2+ metastatic nodules with sensitivity of 100% (114 of 114), specificity of 100% (269 of 269), and accuracy of 100% \((n = 383; \text{Fig. 6B})\). Without injection of Herceptin-RhodG, only red fluorescence signal was detected from Balb/3T3/HER2+/RFP+ tumor sites both on \textit{in situ} postmortem and \textit{ex vivo} spectral fluorescence imaging (Fig. 6C). It is noted that faint green fluorescence contamination was found especially at the rim of the lungs even without injection of Herceptin-RhodG likely related to surrounding collagen (Fig. 6C).

**Discussion**

Accurate localization of pulmonary metastases is an important clinical consideration in improving the outcome of pulmonary metastasectomies (3). The lung is a favorable organ for optical imaging because of its low light attenuation tissue characteristics and because it is readily exposed during thoracoscopy or thoracotomy. Ordinarily, when depth penetration is an issue, near-IR fluorophores are preferred; however, for surface imaging applications, higher quantum yield fluorophores of lower wavelength such as RhodG yield brightly fluorescent images, which enable improved differentiation of the target from the background autofluorescence, allowing the detection of tiny pulmonary nodules.

The lungs are a major site of metastases, which are often a life-limiting manifestation of cancer due to obstruction, bleeding, and infection. Surgical resection has been shown to improve survival; however, recurrence rates within the lungs are as high as 40%. The relatively high miss rate is partly because the lungs are large organs and small metastases can be hidden from view. The surgeon relies on a combination of preoperative

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**Fig. 3.** Herceptin-RhodG produces highly fluorescent dots within the cells. Fluorescence microscopy (left) as well as differential interference contrast (right) images of 3T3/HER2+ cells are shown. Fluorescence microscopy immediately after washing showed high fluorescence on the cell surface with all four dyes. At 8 h, however, after washing the dyes, the fluorescent dots produced by Herceptin-RhodG became larger and brighter than the others. Photographic exposure time: Herceptin-BODIPY, Herceptin-FITC, or Herceptin-OreG = 2 s (at all time points), Herceptin-RhodG (0 h) = 2 s; Herceptin-RhodG (8 h) = 1 s (the latter exposure time is half the others because of saturation of the charge-coupled device camera caused by greatly increased fluorescence with Herceptin-RhodG). Original magnification, \( \times 200 \).
computer-assisted tomography scans, direct visualization, and their tactile skills to localize pulmonary metastases, a paradigm that has changed little in the last 25 years.

We propose a method to enhance the surgical resection of pulmonary metastases. Many metastases express specific cell-surface markers. A commonly overexpressed cell-surface receptor in breast cancer metastases is HER2/neu, which binds epithelial growth factor. The humanized antibody to HER2/neu, trastuzumab (Herceptin; Genentech), is clinically approved for the therapy of breast cancer. We hypothesized that we could use Herceptin as a vector to deliver an optically fluorescent probe to a tumor, thus enhancing it and making it easier to detect during surgery. We showed that Herceptin bound to the fluorophore RhodG revealed HER2/neu-expressing pulmonary metastases but not lesions that did not express this cell-surface marker. We also showed that a nonspecific antibody does not enhance tumors.

Because Herceptin is already clinically approved, translation to the clinic of Herceptin-RhodG conjugate is very feasible. Of course, the toxicity of the conjugate must still be tested, but the low dosage of the antibody-fluorophore conjugate suggests that toxicity will not be an issue. Moreover, because Herceptin is just one of several monoclonal antibodies approved for human use, it is possible that the same technique could be used, either singly or as a cocktail of antibodies to identify pulmonary metastases that display a range of different cell-surface markers. Thus, this method could have broad implications for improving surgical resections in all parts of the body.

The selection of the optimal fluorophore conjugate is important for future clinical translation. Although in vitro results at physiologic pH suggested that Herceptin OncG was the best agent, Herceptin-RhodG proved to have stronger and longer-lasting fluorescence signal at acidic pH, therefore yielding the brightest images after cellular internalization probably because RhodG is a positively charged molecule. Thus, Herceptin-RhodG had the desirable properties of relatively low signal when unbound (at physiologic pH) but showed comparatively higher signal when bound and internalized within HER2+ cells and was therefore selected as the agent of choice for these studies.

**Fig. 4.** Herceptin-RhodG specifically accumulates in 3T3/HER2+ cells. Flow cytometry analysis of Balb/3T3/HER2– cells (A) and 3T3/HER2+ cells (B) at 3 h postincubation with Herceptin-RhodG. A, the MFI values and percent of positive cells of untreated Balb/3T3/HER2– cells (Control) were 4 absorbance units and 2%, respectively, which did not change after incubation with Herceptin-RhodG. B, the MFI values and percent of positive cells of untreated 3T3/HER2+ cells were 7 absorbance units and 2%, respectively, which significantly increased to 825 absorbance units and 100%, respectively, after incubation with Herceptin-RhodG. C, fluorescence microscopy confirmed that Herceptin-RhodG was bound and internalized into 3T3/HER2+ cells whereas HUT-RhodG did not bind or internalize into 3T3/HER2+ cells. Herceptin-RhodG did not bind to Balb/3T3/HER2– cells.
The determination of sensitivity and specificity of optical agents in animal models is a difficult problem because of the small size of the targeted lesions. We approached this problem by developing an animal model of pulmonary metastases in which the targeted tumor could be enhanced by the targeted fluorophore whereas the control tumor would show constitutive fluorescence due to the expression of RFP. By calculating the true positive rate and false positive rate in the same animal, both sensitivity and specificity could be determined. We believe that this animal model may serve as an example for future experiments using targeted fluorophores intended for possible clinical translation. Both parent cells were derived from murine fetal fibroblasts, which were transfected with either HER2 or dsRed genes using the same cytomegalovirus promoter.

Fig. 5. Herceptin-RhodG specifically accumulated in the HER2+ metastatic tumors in the lung. A, a time course series of white light images and spectral fluorescence images of 3T3/HER2+ lung metastases using either HUT-RhodG (right) or Herceptin-RhodG (middle). Balb/HER2+ lung metastases with Herceptin-RhodG (left) are shown as well as the histogram of fluorescence intensities of each lung. Images were taken at 1, 2, 4, and 7 d postinjection of each conjugate. B, white light, unmixed, and composite fluorescence images corresponding to those shown in (A). RhodG (green) and autofluorescence (white) are separately shown on the composite images. C, ex vivo stereomicroscopic and unmixed fluorescence images of lungs bearing 3T3/HER2+ metastatic tumors at 2 d postinjection of HUT-RhodG (left) and Herceptin-RhodG (right). D, ex vivo stereomicroscopic and close-up unmixed fluorescence images of a lung bearing 3T3/HER2+ metastatic tumors at 1 d postinjection of Herceptin-RhodG (two left images) and fluorescence microscopic images (differential interference contrast and green fluorescent images) of a frozen section of the same lung (two right images). Herceptin-RhodG accumulates at the rim of small metastatic 3T3/HER2+ tumors. The findings are consistent with the binding site barrier. The rim accumulation of Herceptin-RhodG in the 3T3/HER2+ tumors (arrows) is also clearly seen in the fluorescent microscopic image.
Another important aspect of experimental design in optical probe experiments is using a control antibody that is isotype matched with the targeting antibody. Non–isotype-matched antibodies could have different performance characteristics based on their isotype alone and not their targeting. We used two Food and Drug Administration–approved humanized antibodies: Herceptin, a CDR–grafted human immunoglobulin G1 antibody against HER2 receptor, and Zenapax (HUT), a CDR–grafted human immunoglobulin G1 antibody against interleukin-2 receptor α-subunit (10, 14). These two antibodies, which were genetically grafted on the same human immunoglobulin G1 framework, differ only in the 12 hypervariable region binding-site peptides and thus should behave pharmacokinetically similar except for their binding characteristics. In addition, neither antibody showed cross reactivity with murine antigens. Therefore, Zenapax (HUT) is an excellent control.

Fig. 6. Two-color in situ postmortem spectral fluorescence imaging shows highly specific accumulation of Herceptin-RhodG into HER2+ tumors. A, two-color in situ postmortem (left) and ex vivo (right) spectral fluorescence images of the left lung bearing both 3T3/HER2+ and Balb/3T3/HER2−/RFP+ tumors at 1 d postinjection of Herceptin-RhodG. The overwhelming majority of red fluorescent sites do not colocalize with green fluorescent sites. B, fluorescence microscopic images of the lung depicted in (A) show no colocalization of red and green fluorescence in any metastatic tumors. C, two-color in situ postmortem (left) and ex vivo (right) spectral fluorescence images of the right lung bearing both 3T3/HER2+ and Balb/3T3/HER2−/RFP+ tumors without injection of Herceptin-RhodG. No green fluorescence is present in any metastatic tumor and some large tumors are shown as defects of both RFP and lung autofluorescence (arrows).
antibody for Herceptin because it is isotype matched and shows >98% protein sequence homology with Herceptin.

The binding site barrier effect was readily identified in these experiments. Herceptin is an antibody with high affinity (Kd 4 nmol/L) for the HER2 antigen. When high-affinity antibodies accumulate around tumors that highly express the cognate antigen, the antibodies are often trapped at the rim of tumor nodules (9) probably because angiogenesis and antibody leakage is highest at the rim and subsequent penetration into the tumor center is slow because of elevated interstitial pressure. Therefore, high-affinity antibodies tend to be trapped at the rim of the tumor and slowly penetrate to the core only after the rim antigens are completely saturated. In this study, Herceptin-RhodG was found in the rim of the tumor at early time points after injection, peaking at 1 day. Whereas the binding site barrier is of concern from a therapeutic perspective, peak nodular fluorescence, from a diagnostic point of view, corresponds to the time of maximal binding barrier effect. Because light is absorbed with depth, the rim enhancement of the tumor is actually an advantage for optical imaging because the optical probe is confined to the surface of the lesion, reducing internal absorption. Therefore, the rim enhancement at early time points is a sign of strong binding affinity and actually assists in vivo fluorescence imaging.

Less than 4% of Balb/3T3/HER2— seemed to bind to Herceptin-RhodG, resulting in a small false positive rate. Twenty three of these 27 tumors were located on the edge of the lung. The edge of the lung had green fluorescence signal even before injection of Herceptin-RhodG probably due to collagen in the adjacent folded pleura. We believe that the false-positive cases arose from small RFP-positive tumors located at the lung edge, which had contamination from natural green fluorescence due to adjoining collagen. It is possible in some cases that two tumors of differing cell type were practically superimposed on each other and the better penetration of the red light of RFP compared with the green light from RhodG led to the impression that RhodG arose from the RFP-expressing tumor. Four of the false-positive tumors could be explained in this way. When thin section tissues were examined by fluorescence microscopy, there were no instances where green and red fluorescence coexisted in the same tumor. In any case, the low false positive rate (~4%) is certainly acceptable given the high sensitivity.

In conclusion, we successfully developed a targeted spectral fluorescence imaging method to visualize HER2+ submillimeter lung metastatic tumors based on the antibody conjugate Herceptin-RhodG. Optical probes based on this and other antibodies may one day assist surgeons in localizing metastatic tumors during surgical resection.

References
Spectral Fluorescence Molecular Imaging of Lung Metastases Targeting HER2/neu
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