Rapid Accumulation and Internalization of Radiolabeled Herceptin in an Inflammatory Breast Cancer Xenograft with Vasculogenic Mimicry Predicted by the Contrast-enhanced Dynamic MRI with the Macromolecular Contrast Agent G6-(1B4M-Gd)\textsubscript{256}

Hisatka Kobayashi, Kazuo Shirakawa, Satomi Kawamoto, Tsunji Saga, Noriko Sato, Akira Hiraga, Ichiro Watanabe, Yuji Heike, Kaori Togashi, Junji Konishi, Martin W. Brechbiel, and Hiro Wakasugi

ABSTRACT

The rapid blood flow and perfusion of macromolecules in the inflammatory breast cancer xenograft (WIBC-9), which exhibits a “vasculogenic mimicry” type of angiogenesis without the participation of endothelial cells and expresses high levels of the HER-2/neu antigen, was evaluated in mice using 3D-micro-MR angiography using a novel macromolecular MR contrast agent (G6-(1B4M-Gd)\textsubscript{256}). Herceptin, which recognizes the HER-2/neu antigen and has similar size (10 nm) to G6-(1B4M-Gd)\textsubscript{256}, accumulated and internalized in the WIBC-9 tumors more quickly than in the control MC-5 tumors that progress with normal angiogenesis. Three-dimensional micro-MRI with the G6-(1B4M-Gd)\textsubscript{256} macromolecular MRI contrast agent distinguishes between the different types of angiogenesis and is predictive of the rapid accumulation and internalization of Herceptin in the WIBC-9 inflammatory breast cancer xenograft.

INTRODUCTION

One of the major problems for antibody-mediated cancer therapy with radiolabeled antibodies and immuno toxins is their slow delivery from the circulation to the target cancer cells (1–3). This slow delivery is a source of toxic side effects to bone marrow, lung, and other organs (4–6). To enhance perfusion into cancerous tissue, chemically or genetically engineered small variants of antibodies have been evaluated with limited success (7–10). Therefore, intact antibodies are still being used in clinical cancer therapies (4, 6, 11–13).

Recently, several investigators have reported a new type of vasculogenesis called vasculogenic mimicry in IBC\textsuperscript{2} osteosarcoma, and melanoma, wherein tumors can obtain nutrients by using alternative pathways without participation of endothelial cells (14–20). The vasculogenic mimicry was originally reported by Maniotis et al. (21) in aggressive melanoma that lacks evidence of significant necrosis and contains patterned networks of interconnected loops of extracellular matrix. The molecular basis for this vasculogenic mimicry in breast cancer has been reported (14, 22). The generation of microvascular channels by genetically deregulated, aggressive tumor cells was termed vasculogenic mimicry to emphasize their de novo generation of vascular channels without the participation of endothelial cells and the independence to angiogenesis. The significant blood flow in the vasculogenic mimicry has also been evaluated by the dynamic micro-MRI study (23). The presence of the endothelial cells, which retards the perfusion of macromolecular drugs into tumor tissue, has been one of the major problems in antibody-mediated therapies. In the current study, the syngeneically xenografted tumor of IBC with vasculogenic mimicry was found to have significant flow between cancer cells without endothelial cells and to exhibit rapid perfusion of macromolecules as determined by the contrast-dynamic micro-MRI using G6-(1B4M-Gd)\textsubscript{256}, a macromolecular MR contrast agent of similar molecular size to IgG, ~10 nm in diameter (24–29). Additionally, this vasculogenic mimicry with rapid perfusion was advantageous for the delivery of radiolabeled Herceptin, a Food and Drug Administration-approved humanized IgG against HER-2/neu, and permitted rapid access of the radioimmunoconjugate to the cancer cells.

MATERIALS AND METHODS

Herceptin. Herceptin, a humanized monoclonal antibody against the HER-2/neu antigen, was kindly provided by Dr. Thomas A. Waldmann, (Metabolism Branch, National Cancer Institute, NIH, Bethesda, MD).

Dendrimer. G6 (Aldrich Chemical Co., Milwaukee, WI) with an ethylenediamine core and 256 reactive exterior amino groups (M, 57,991) was used for the preparation of the MRI contrast agent.

Establishment of WIBC-9 and MC-5. Tumor specimens from the patients with IBC and non-IBC were obtained immediately after surgery and processed as reported previously (30). The tumor xenografts were subsequently transplanted as described previously (14). This set of animal protocol experiments was approved by the Animal Use Committee of the National Cancer Center.

Conjugation of Chelates to Herceptin. Herceptin was concentrated to ~5 mg/ml and diafiltered against 0.1 M PB at pH 9.0 with Centricon 50 (Amicon, Inc., Beverly, MA). The Herceptin was reacted with a 20-fold molar excess of 1B4M as described previously (31, 32).

Radiolabeling of Herceptin. Carrier-free 111InCl\textsubscript{3} was a gift from Japan Mediphysics (Nishinomiya, Japan), and 125I was purchased from NEN DuPont (Boston, MA). Herceptin conjugated with 1B4M was reacted with 111In in 0.3 M sodium acetate buffer at pH 6.0 for 15 min at room temperature as described previously (31).

Herceptin was labeled with 125I by a modified chloramine-T method as described previously (32, 33). The specific activity of the radiiodinated antibody was 7 mCi/mg. Purity was >98% as determined by size-exclusion high-performance liquid chromatography using a TSK G2000SW column (TosoHaas, Philadelphia, PA; 0.1 M PBS; 0.01 M KCl; pH 7.4; 1 ml/min).

Immunoreactivity and Cell-binding Ability. The immunoreactivity of the radiolabeled Herceptin was determined using a cell-binding assay described previously (33). In brief, aliquots of 111In-labeled or 125I-labeled Herceptin (3 ng/100 µl) were incubated for 2 h at 4°C with ~1 × 10\textsuperscript{7} WIBC-9 or MC-5 cells, which were obtained from the xenografted tumors. The cell-bound radioactivity was separated by centrifugation and counted in a gamma counter. Nonspecific binding to the cells was examined under conditions of antibody excess (50 µg of nonradiolabeled Herceptin). The specific binding was calculated as the percentage of binding subtracting the nonspecific binding.

Preparation of Macromolecular MR Contrast Agent for MR Angiography. The preparation of the G6-(1B4M-Gd)\textsubscript{256} macromolecular MR contrast agent (M, 240,000) was described previously (25, 26). In brief, the G6...
dendrimer was concentrated to 5 mg/ml and diafiltered against a 0.1 M sodium phosphate buffer at pH 9.0 reacted with a 512-fold molar excess of 1B4M at 40°C and maintained at pH 9.0 with 1 M NaOH for 24 h. G6–1B4M256 conjugate (3 mg; containing 4 μmol 1B4M) was mixed with nonradioactive Gd(III) citrate (6 μmol) in 0.3 M ammonium citrate buffer for 2 h at 4°C. The excess Gd(III) was removed by diafiltration using the Centricon 30 (Amicon Co.) while simultaneously changing the buffer to 0.05 M PBS.

Biodistribution of Radiolabeled Herceptin in WIBC-9 Tumor-bearing Mice. Four groups of mice (n = 4–5) bearing either WIBC-9 or MC-5 tumor xenografts, were injected i.v. with both 2.5 μCi of 125I-labeled Herceptin and 0.8 μCi of 111In-labeled Herceptin. The mice were euthanized at 3 h, 1 d, 2 d, or 4 d after injection. Their blood was drawn and aliquoted for counting, and then their organs were harvested and counted in a gamma counter (Aloka, Mitaka, Japan). The data were expressed both as a percentage of injected dose per gram of normal tissues and tumors; bars, ±SD (n = 4 or 5).

Dynamic Contrast Enhanced Micro-MRI of Tumor-bearing Mice with Macromolecular MR Contrast Agent. To evaluate the hemodynamics and perfusion of a macromolecule of similar size to Herceptin in these tumor models, dynamic micro-MRI of the tumor-bearing mice were obtained by injection of 0.03 mmol Gd/kg of G6-(1B4M-Gd)256 using a 1.5-Tesla superconductive magnet unit (Signa, General Electric Medical Systems, Milwaukee, WI), as described previously (34, 35). Six female 8-week-old BALB/c-nu/nu mice bearing WIBC-9 (n = 3) or MC-5 (n = 3) xenografts were used. For the dynamic study, the three-dimensional fast spoiled gradient echo technique [repetition time (TR)/echo time (TE) 10.5/2.7; TI 31 msec; flip angle 30°; scan time 29 s; 1 number of excitation (NEX)] with chemical fat-suppression was used for all of the mice. The images were acquired at 30 s intervals at preinjection from 0 (immediately after injection) to 4.5 min after injection of the contrast agent. The coronal images were reconstructed with a 0.8-mm section thickness and with a 0.4-mm overlap. The field of view was 8 × 4 cm, and the size of matrix was 256 × 128. The delayed coronal MR images were obtained with the three-dimensional fast spoiled gradient echo technique (TR/TE 10.5/2.7; TI 31 ms; flip angle 30°; scan time 1 min 30 sec; 3NEX) with chemical fat-suppression used for all of the mice at 5 min postinjection immediately after the dynamic study. Additionally, the slice data were processed subtracting from postcontrast images to precontrast

Fig. 1. Biodistribution of 111In-labeled Herceptin in nude mouse xenografted with WIBC-9 (A) and MC-5 (B) tumors at 3 h (?), 1 day (□), 2 days (■), and 4 days (□) after injection. The data are expressed as the mean percentages of injected dose per gram of normal tissues and tumors; bars, ±SD (n = 4 or 5).

Fig. 2. Biodistribution of 125I-labeled Herceptin in nude mouse xenografted with WIBC-9 (A) and MC-5 (B) tumors at 3 h (?), 1 day (□), 2 days (■), and 4 days (□) after injection. The data are expressed as the mean percentages of injected dose per gram of normal tissues and tumors; bars, ±SD (n = 4 or 5).

Fig. 3. Plots of the accumulation of 111In-labeled and 125I-labeled Herceptin in WIBC-9 tumor at 3 h (?), 1 day (□), 2 days (■), and 4 days (□) after injection. The data are expressed as the mean percentage of injected dose per gram of WIBC-9 tumor; bars, ±SD (n = 4 or 5). *, significant differences (P < 0.01).
images and constructed into three-dimensional MR angiograms using the maximum intensity projection method using the Advantage Windows (General Electric Medical Systems).

Scintigraphic Study. Scintigraphic imaging of the nude mice injected with $^{111}$In-labeled Herceptin was performed; the mice, which were examined with dynamic micro-MRI using G6-(1B4M-Gd)$_{256}$, were injected i.v. with 100 $\mu$Ci/28 $\mu$g of $^{111}$In-labeled Herceptin. A gamma camera (Hitachi Medical Co., Tokyo, Japan) equipped with a 4-mm pin-hole collimator was used to perform scintigraphy at 3 h, 1 day, 2 days, and 4 days after injection. The mice were anesthetized with 1.15 mg of sodium pentobarbital (Dainabot, Osaka, Japan) per mouse. Imaging data were recorded in a 128-by-128 byte mode matrix using a 60% window centered over the 250 keV energy peak of $^{111}$In. Images were acquired for 200,000 counts.

Histological Comparison with MRI and Scintigraphic Images. The mice were killed with an injection of 10 mg of sodium pentobarbital via the tail vein immediately after the final series of examinations and fixed in formaldehyde for >2 weeks. The tumors were sectioned in the same coronal planes as the MR sectional images, examined by stereoscopic microscopy (Photomakroskop; Wild, Heerbrugg, Switzerland), and correlated with the serial MR images. Additionally, the coronal histological sections of the tumors stained with H&E were correlated with the MR images. All of the studies reported herein were approved by the Animal Care Committee of Kyoto University.

Statistical Analysis. Statistical analysis was performed using Student’s $t$ test (StatView; SAS Institute Inc., Cary, NC).

RESULTS

Immunoreactivity and Cell-binding Ability. The maximum specific binding of the $^{111}$In-labeled Herceptin to the WIBC-9 and the MC-5 cells was 82% and 18%, respectively. The maximum specific binding of the $^{125}$I-labeled Herceptin to the WIBC-9 and the MC-5 cells was 78% and 16%, respectively. Ninety-five percent of their binding was inhibited by the addition of 50 $\mu$g of nonradiolabeled Herceptin.

Biodistribution of Radiolabeled Herceptin in WIBC-9 Tumor-bearing Mice. The biodistribution of radiolabeled Herceptin in the normal organs exhibited no significant differences at most time points between mice bearing either WIBC-9 or MC-5 tumors (Figs. 1 and 2). However, both $^{111}$In- and $^{125}$I-labeled Herceptin accumulated in the WIBC-9 tumors significantly higher than in the MC-5 tumors at 3 h after injection ($P < 0.01$). Although the $^{111}$In-labeled Herceptin
accumulated in the WIBC-9 tumors significantly higher than in the MC-5 tumors up to 4 days after injection, the accumulation of 125I-labeled Herceptin in the WIBC-9 tumor decreased at 1 day after injection and later was significantly lower than that of the 111In-labeled Herceptin (Fig. 3). The tumor:normal tissue ratio in all of the organs was significantly higher in mice bearing WIBC-9 tumors than in mice bearing MC-5 tumors, especially at 3 h after injection (Fig. 4).

Dynamic Contrast Enhanced Micro-MRI of Tumor-bearing Mice with Macromolecular MR Contrast Agent. The serial dynamic MRI using the macromolecular contrast agent G6-(1B4M-Gd)256 showed a more rapid and more diffuse enhancement of the WIBC-9 tumor than that of the MC-5 tumor (Fig. 5). Both two-dimensional micro-MRI (Fig. 6, a and b) and three-dimensional dynamic micro-MR angiography (Fig. 6, c and d) of tumor-bearing nude mice using the macromolecular contrast agent G6-(1B4M-Gd)256, which had similar size to that of Herceptin, showed significantly better diffusion and early contrast enhancement in the WIBC-9 tumor than in the MC-5 tumor as indicated by the micro-MR angiograms.

Scintigraphic Study. The scintigrams of tumor-bearing mice indicated high homogeneous accumulation of the 111In-labeled Hercept-
tin in the WIBC-9 tumors within 3 h after injection (Fig. 7) compared with that in the MC-5 tumor. The WIBC-9 tumor-to-background contrast improved at later time points after injection. These results were consistent with the biodistribution data shown in Figs. 1 and 2.

**Histological Comparison with MRI and Scintigraphic Images.** Numerous RBCs existing in the channel components, called vasculogenic mimicry, were shown to be present especially at the inner part of the WIBC-9 tumor (Fig. 8a). This correlates with the rapid, diffusion contrast enhancement on the dynamic MRI with G6-(1B4M-Gd)256 macromolecular contrast agent (Fig. 6, a and c) and the quick accumulation of the 111In-labeled Herceptin in the scintigraphic image obtained at 3 h after injection (Fig. 7a). However, this finding of vasculogenic mimicry was sometimes misread by pathologists as an artifact or subtle intratumoral hemorrhage. In contrast, the MC-5 tumor showed the central necrotic foci. In addition, the capillaries in the MC-5 tumor were lined with endothelial cells. This correlates with the absent contrast enhancement on the dynamic MRI with G6-(1B4M-Gd)256 macromolecular contrast agent (Fig. 6, b and d) and the lack accumulation of the 111In-labeled Herceptin in the scintigraphic image obtained at 3 h after injection (Fig. 7b).

**DISCUSSION**

HER-2 is a membrane receptor of which the overexpression is strongly associated with poor prognosis in breast cancers (36, 37). Inhibition of HER-2 activity can reduce or inhibit tumor growth, which led to the development of Herceptin, a Food and Drug Administration-approved recombinant humanized anti-HER-2 monoclonal antibody now in clinical use (38–40). However, the objective response rate to Herceptin monotherapy is actually quite low (41). Therefore, Herceptin armed with cytotoxic radionuclides of other cytotoxic agents to augment the biological activity and to capitalize on the selective delivery action is a promising therapeutic approach.

One of the major problems in the use of whole IgG for cancer therapy is slow perfusion into tumor tissue because of both size and competing catabolism. In regard to the perfusion of macromolecules like an IgG, vasculogenic mimicry-like angiogenesis, a condition in which the tumor vascular channels are not lined by endothelial cells, is advantageous. In the present study, radiolabeled Herceptin accumulated to the xenografted WIBC-9 tumors more rapidly and higher than to the MC-5 tumors, and a clear scintigraphic image could be obtained as early as 3 h after injection of 111In-labeled Herceptin. The discrepancy between the accumulation of the 111In-labeled and the 125I-labeled antibody was reported to be a consequence of the internalization of the antibodies and subsequent dehalogenation (9, 42). The high accumulation of both 111In-labeled and 125I-labeled Herceptin at 3 h after injection and the discrepant accumulation between them at 1 day after injection provide evidence that the Herceptin quickly accumulated and was internalized into the WIBC-9 cells. Therefore, the WIBC-9, which is characterized as a tumor accompanied with the formation of vascular channels without endothelial cells, might be an optimal target for developing antibody-based therapies with radiometal-labeled or toxin-conjugated Herceptin rather than antibody-dependent cellular cytotoxicity induced by Herceptin itself because of this rapid internalization.

In addition, although scintigraphy with radiolabeled albumin or antibodies is the only available method to evaluate the perfusion of macromolecules, spatial resolution is quite limited. In this study, high-resolution micro-MRI with a macromolecular contrast agent, G6-(1B4M-Gd)256 (25, 26) was used to evaluate the perfusion of the macromolecule. In the current study as well as in previous studies, three-dimensional micro-MRI of tumors with well-known types of
vasculogenesis lined by endothelial cells such as those obtained from the MC-5 and LS180 cell lines depicted only continuous vascular structure and no diffuse enhancement in the tumors within 5 min after injection of G6-(1B4M-Gd)256 (35). The diffuse enhancement in the tumor tissue was shown only in the WIBC-9 tumor by the three-dimensional micro-MRI within 5 min after injection. These diffuse enhancement areas as indicated by MR corresponded well to the areas where radiolabeled Herceptin accumulated. These findings in the WIBC-9 tumors are also compatible and indicative of the special type of angiogenesis termed vasculogenic mimicry, a type of vasculogenesis without the involvement of endothelial cells; thus no barrier exists for Herceptin to efficiently access these cancer cells (14).

In conclusion, contrast-enhanced dynamic micro-MRI with a G6-(1B4M-Gd)256 macromolecular MR contrast agent was able to evaluate the intratumoral perfusion of macromolecules because of different types of angiogenesis and was predictive of the rapid accumulation and internalization of Herceptin in the IBC xenograft WIBC-9.

ACKNOWLEDGMENTS

We thank Dr. Thomas A. Waldmann of the Metabolism Branch, National Cancer Institute, NIH, for kindly providing the Herceptin for this study and for assistance in the preparation of this manuscript.

REFERENCES


Fig. 8. Stereoscopic (×10) and light (×40 and ×400) microscopy pictures of WIBC-9 (a) or MC-5 (b) tumors. WIBC-9 showed the solid white tumor without necrotic foci and numerous small vascular channels without lining endothelial cells, which corresponded to vasculogenic mimicry. In contrast, MC-5 showed the white tumor with central necrotic foci. All capillaries in MC-5 tumors were lined by the endothelial cells.
Rapid Accumulation and Internalization of Radiolabeled Herceptin in an Inflammatory Breast Cancer Xenograft with Vasculogenic Mimicry Predicted by the Contrast-enhanced Dynamic MRI with the Macromolecular Contrast Agent \( \text{G6-(1B4M-Gd)}_{256} \)

Hisataka Kobayashi, Kazuo Shirakawa, Satomi Kawamoto, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/3/860

Cited articles
This article cites 40 articles, 17 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/3/860.full#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/62/3/860.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.