ABSTRACT

In the present study, we examined hemodynamics in vasculogenic mimicry (VM) and angiogenesis of inflammatory breast cancer (IBC) xenografts (WIBC-9), having previously reported on the unique histological features and molecular basis of these processes (K. Shirakawa et al., Cancer Res., 61: 445–451, 2001). Histologically, the WIBC-9 xenografts exhibited invasive ductal carcinoma with a hypervascular structure (angiogenesis) in the tumor margin and VM without endothelial cells, central necrosis, or fibrosis in the tumor center. Results of molecular analysis indicated that WIBC-9 had a vasculogenic phenotype, including expression of Flt-1 and Tie-2. Comparison of WIBC-9 with an established non-IBC xenograft (MC-5), using time-coursed dynamic micromagnetic resonance angiography analysis (with our newly developed intravascular macromolecular magnetic resonance imaging contrast agent), electromicroscopy, and immunohistochemistry, demonstrated blood flow and a VM-angiogenesis junction in the central area of the WIBC-9 tumor. It has previously been considered impossible to prove a connection between VM and angiogenesis using angiography, because there are no intravascular macromolecular magnetic resonance imaging contrast agents that do not exhibit significant leakage through the vascular wall. In the present study, laser-captured microdissection was performed in regions of WIBC-9 tumors that exhibited VM without endothelial cells, central necrosis, or fibrosis, revealing expression of human-Flt-1 and human-Tie-2 and the absence of human-CD31, human-endothelin B receptor, and human-thrombin receptor. These facts led us to hypothesize that the VM of WIBC-9 involves hemodynamics that serve to feed WIBC-9 cells, and this in turn suggests a connection between VM and angiogenesis.

INTRODUCTION

Tumors require a blood supply for growth and hematogenous metastases. Most attention has been focused on the role of angiogenesis, the recruitment of new vessels into a tumor from preexisting vessels. Previously, we and others reported the presence of VM [a condition in which tumors (IBC and melanoma) may have the potential to feed themselves without angiogenesis] in the tumor-bearing state (1–7). The interface between erythrocytes and tumor cells in VM has been visualized by electromicroscopy and immunohistochemistry (1). Using semiquantitative RT-PCR with species-specific primers, we have found a molecular basis for VM (1). The generation of microvascular channels by genetically deregulated, aggressive tumor cells has been termed “vasculogenic mimicry” to emphasize their de novo generation, without participation of ECs and independent of angiogenesis. This mechanism, by which an aggressive tumor generates its own network of pseudo-vascular channels, may challenge the assumption that angiogenesis and related mechanisms are the only means by which a tumor acquires a blood supply. However, the hemodynamics of VM have never been observed. In this study, we investigated the hemodynamics of VM and angiogenesis of IBC, using WIBC-9 xenografts and dynamic micro-MRA analysis with our newly developed intravascular macromolecular MRI contrast agent. The results of LCM in the VM regions of WIBC-9 strongly suggest the existence of hemodynamics that serve to feed WIBC-9 cells, and this in turn suggests a connection between VM and angiogenesis.

MATERIALS AND METHODS

Establishment of WIBC-9, MC-5, MC-2, and MC-18. The animal protocols for all experiments were approved by the Animal Use Committee of the National Cancer Center. Tumor specimens from patients with IBC and non-IBC cancer were obtained immediately after surgery and processed as reported previously (1). The tumor xenografts were subsequently serially transplanted over a period of more than 3 years, with up to 15 transplants, and stable serially transplantable xenografts (WIBC-9, MC-5, MC-2, and MC-18) were successfully established in BALB/c nude mice (CLEA Japan, Tokyo, Japan; Refs. 1). When the tumors had reached 10 mm in diameter, they were examined by dynamic micro-MRA.

Dynamic Micro-MRA with an Intravascular Contrast Agent. We performed dynamic micro-MRA analysis, using our newly developed intravascular macromolecular MRI contrast agent [G6D-(1B4M-Gd)256 (M0, 240,000), which consistently showed no significant leakage through the vascular wall after remaining in circulation for more than 30 min] to evaluate the physiological properties of the vascular channels in the xenografted tumors. The methods used for synthesis and preparation of the contrast agent and for obtaining dynamic MR images, along with a discussion of the agent’s properties in relation to visualizing microvasculature, have previously been published (8). MR angiography of the mice was performed with injection of 0.066 mmol Gd/kg of Gd(6-(1B4M-Gd)256 using a 1.5-tesla superconductive magnet unit (Signa; General Electric Medical System, Milwaukee, WI). We used female 8-week-old BALB/c nude mice bearing either WIBC-9 or MC-5 tumor xenografts. All of the images were obtained with dual phased-array 3-inch round surface coils, fixed at 3-cm intervals by an in-house-constructed mouse and coil holder. The mice were anesthetized with 1.15 mg of sodium pentobarbital (Dinabot, Osaka, Japan), and placed at the center of the coils. The three-dimensional-fast spoiled gradient echo technique (efgref3d; TR/TE, 10.5/ 2.7; flip angle, 30°) with chemical fat-suppression was used for all mice. The images were acquired before injection of the contrast agents and at 0 (immedi-ately postinjection), 1, 2, 3, 5, 8, 10, 15, and 30 min postinjection. The coronal images were reconstructed with 1.0-mm section thickness and 0.5-mm overlap. The FOV was 8 × 4 cm, and the size of the matrix was 256 × 128. This procedure was performed with mice bearing WIBC-9 and MC-5 tumors (n = 3, for each).

Morphological Analysis and Immunohistochemistry. In addition to dy-namic micro-MRA, we performed the following analyses on the specimens,
using conventional methods: H&E staining, Giemsa staining and electron microscopy. Frozen and paraffin sections of the nonresected tumor samples that had been examined by dynamic micro-MRA were embedded in O.C.T. Compound (Mile’s Sankyo, Tokyo, Japan), and assayed using the immunoperoxidase procedure. Antimurine CD31 (Pharmingen, San Diego, CA) was used as the primary antibody. The reaction was visualized using streptavidin-biotin (Pharmingen) techniques.

LCM. Paraffin-embedded WIBC-9 and MC-5 xenograft specimens were cut into serial 5-μm sections and mounted on uncoated slides treated with diethyl pyrocarbonate. The VM in WIBC-9 and MC-5 cancer cells was examined in coverslipped H&E-stained sections. One specimen was selected for collection of 2000 individual cells for mRNA isolation and nested PCR examination in coverslipped H&E-stained sections. One specimen was selected cut into serial 5-μm sections and mounted on uncoated slides treated with diethyl pyrocarbonate. Antimurine CD31 (Pharmingen, San Diego, CA) and a 7.5-μm laser beam at 50 mV. Target cells in the specimens were easily identified and captured; tumor cells immediately adjacent to necrotic areas and blood cells were avoided.

RT-PCR Conditions and Reagents. Total RNA was isolated from LCM-collected cells and culture cell lines (SK-BR-3, MCF-7 and HUVEC; American Type Culture Collection, Manassas, VA, and Sankojunyaku Co., Tokyo, Japan) using the guanidinium thiocyanate-phenol-chloroform extraction method. PCR was designed to amplify specific mRNAs, using published sequences. Primer sequences for VEGF were as follows: sense, 5'-GGCCGACCATGGGAGGA-3'; antisense, 5'-TGCAGGACACTTTCACCG-3' (amplicon size, 157 bp). Primers for Flt-1 were as follows: sense, 5'-TITTTACCGGACCGCTCT-3'; antisense, 5'-GGGTGCTAGCTGGATGTCTT-3' (amplicon size, 159 bp). Nested primers for Flt-1 were as follows: sense, 5'-GGGCGACGAGCCACGTCT-3'; antisense, 5'-GCCGCCGTCCTCTTATT-3' (amplicon size, 122 bp). Primers for KDR were as follows: sense, 5'-CAACCAATATTACTCCTAC-3'; antisense, 5'-GCAAATCAAAATCTGCTGC-3' (amplicon size, 180 bp). Nested primers for KDR were as follows: sense, 5'-TTGTGCGTCTGTAAGGGCTCA-3'; antisense, 5'-GACTCTAAATCTCTGCTAC-3' (amplicon size, 125 bp). Primers for Ang-1 were as follows: sense, 5'-ACCCAGGCTTACAGTGAT-3'; antisense, 5'-CAAGCATCAACACACTGAT-3' (amplicon size, 180 bp). Primers for Ang-2 were as follows: sense, 5'-TTCAAGACATCTCCAGGTAC-3'; antisense, 5'-CCCTGACACCACGACAC-3' (amplicon size, 132 bp). Nested primers for Ang-2 were as follows: sense, 5'-TCTAACATCCTCACGTCAC-3'; antisense, 5'-CCGCTGACGGGACGACACGAC-3' (amplicon size, 131 bp). Primers for Tie-2 were as follows: sense, 5'-GACTCCAGGAGTCAGAGTG-3'; antisense, 5'-CAATCTCAAGGAGCGAGT-3' (amplicon size, 177 bp). Nested primers for Tie-2 were as follows: sense, 5'-TGCGGCTGAGGTCGTTCATG-3'; antisense, 5'-CAAGTCATCCGGCACTGAG-3' (amplicon size, 127 bp). Primers for CD31 were as follows: sense, 5'-AGGGTTTATCTGCGAAGTGT-3'; antisense, 5'-TCATTATGCTGTACCTC-3' (amplicon size, 222 bp). Nested primers for CD31 were as follows: sense, 5'-AGGTGTTTATCTGCGAAGTGT-3'; antisense, 5'-TCATTATGCTGTACCTC-3' (amplicon size, 222 bp). Nested primers for CD31 were as follows: sense, 5'-AGGTGTTTATCTGCGAAGTGT-3'; antisense, 5'-TCATTATGCTGTACCTC-3' (amplicon size, 222 bp). Nested primers for CD31 were as follows: sense, 5'-AGGTGTTTATCTGCGAAGTGT-3'; antisense, 5'-TCATTATGCTGTACCTC-3' (amplicon size, 222 bp). Nested primers for CD31 were as follows: sense, 5'-AGGTGTTTATCTGCGAAGTGT-3'; antisense, 5'-TCATTATGCTGTACCTC-3' (amplicon size, 222 bp). Nested primers for CD31 were as follows: sense, 5'-AGGTGTTTATCTGCGAAGTGT-3'; antisense, 5'-TCATTATGCTGTACCTC-3' (amplicon size, 222 bp). Nested primers for CD31 were as follows: sense, 5'-AGGTGTTTATCTGCGAAGTGT-3'; antisense, 5'-TCATTATGCTGTACCTC-3' (amplicon size, 222 bp). Nested primers for CD31 were as follows: sense, 5'-AGGTGTTTATCTGCGAAGTGT-3'; antisense, 5'-TCATTATGCTGTACCTC-3' (amplicon size, 222 bp). Nested primers for CD31 were as follows: sense, 5'-AGGTGTTTATCTGCGAAGTGT-3'; antisense, 5'-TCATTATGCTGTACCTC-3' (amplicon size, 222 bp).

PCR was performed using the following generic annealing temperatures: 50°C for VEGF, Ang-1, endothelin B receptor, CD31, and Ang-2; 54°C for Flt-1 and Tie-2; 51°C for KDR; 52°C for thrombin receptor; and 57°C for G3PDH. PCR was performed with 40 cycles, as follows: 95°C for 30 s, gene-specific annealing temperature for 15 s, 72°C for 30 s. Additionally, agarose gel electrophoresis of the PCR products, followed by staining with ethidium bromide, was performed to confirm the specificity of the amplification.

Comparative Analyses. The following analyses were performed on WIBC-9 MC-5, MC-2, MC-18, HUVEC, SK-BR3, and MCF-7, to determine patterns of VM and angiogenesis: dynamic MRA, histopathology, and post-LCM RT-PCR.

Statistical Analysis. All data are expressed as the mean ± SD. StatView computer software (ATMS Co., Tokyo, Japan) was used for statistical analysis of results for WIBC-9 and MC-5. Two-sided Ps less than 0.05 were considered to indicate statistical significance.

RESULTS

Horizontal Scanning of WIBC-9 and MC-5 by Dynamic Micro-MRA with an Intravascular Macromolecular MRI Contrast Agent. To visualize blood flow in VM and neovascular vessels (angiogenesis) of the xenografted tumor, we used dynamic micro-MRA analysis with our newly developed intravascular macromolecular MRI contrast agent [G6D-(1B4M-Gd)256 (M, 240,000)]. In Fig. 1, we show 2-mm-interval horizontal scans of the ventral area of anesthetized xenografted mice. To compare tumor signal intensities between mice, the amount of G6D-(1B4M-Gd)256 was normalized to 0.066 mmol Gd per kg of mouse body weight. Loupe images and high-power H&E staining images were also used for comparison, along with a side-by-side comparison of the MRA data. The tumor marginal area of WIBC-9 and MC-5 exhibited a high-intensity signal that completely surrounded the xenografted tumor, a result consistent with angiogenesis (compare Fig. 1, A–E, with Fig. 1, F–J). The high-power H&E staining images clearly showed neovascular vessels (compare Fig. 1, D and E, with Fig. 1, I and J). In the tumor center, WIBC-9 exhibited multiple high-intensity spots (+), a finding consistent with pathological VM (Figs. 1, A–C, and 4), whereas MC-5 exhibited a low-intensity signal or a lack of signal (+), a finding consistent with central necrosis and disappearance of nuclei (Figs. 1, F–H, and 4; compare circled areas of Fig. 1, B–E with those of Fig. 1, G–J).

Time-coursed MRA of WIBC-9 and MC-5. Time-coursed micro-MRA was performed to analyze hemodynamics in VM and angiogenesis. The images were acquired before injection of the contrast agents and 0 (immediately postinjection), 1, 2, 3, 5, 10, 15, and 30 min postinjection. The tumor marginal area of WIBC-9 and MC-5 exhibited a signal that gradually increased in intensity, a result consistent with time lag relative to the intensity recorded for the heart (which reflects properties of blood) and the liver (a highly vascularized organ). In the tumor center, WIBC-9 tumors exhibited spots in which the signal increased in intensity (which is consistent with the intensity observed at the tumor margin), whereas MC-5 tumors exhibited a low-intensity signal or a lack of signal (compare circled areas of Figs. 2, A–H with those of Fig. 2, I–P). All of the data were obtained directly from the MRA analyzer and are shown in the graphs in Fig. 3.

Hemodynamics of VM and Angiogenesis of IBC and Non-IBC Xenografts. To analyze hemodynamics in VM and in angiogenesis, we gated on three ROI in the central area and the marginal area of the xenografted tumors and counted time-coursed pixel numbers per mm². Three experiments were performed on these three gated ROI. All of the data in Fig. 3 were obtained directly from the MRA analyzer and are expressed as the mean ± SD. To compare tumor signal intensities between mice, the amount of contrast agent per mouse was normalized, resulting in normalization of heart signal intensities between mice. The time-coursed intensity of the tumor center (which

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Fig. 1. Horizontal scanning of WIBC-9 and MC-5 by dynamic micro-MRA with an intravascular macromolecular MRI contrast agent. Shown are 2-mm-interval vertical sections of the xenografted mice with loupe images and high-power H&E staining images. The tumor marginal area of WIBC-9 and MC-5 exhibited a high-intensity signal that completely surrounded the xenografted tumors, a result consistent with angiogenesis (compare A–E with F–J). In the tumor center, WIBC-9 exhibited multiple high-intensity spots (+) consistent with histological VM, whereas MC-5 exhibited a low-intensity signal or a lack of signal (++) consistent with central necrosis (compare circled areas of B–E with those of G–J).
corresponded to the hemodynamics of VM, as shown in Figs. 1 and 4) was consistent with the time-coursed intensity of the tumor margin (which corresponded to the hemodynamics of angiogenesis, as shown in Fig. 5). Examination of the hemodynamics of VM revealed blood flow with two peaks of intensity and a statistically significant time lag relative to the hemodynamics of angiogenesis. The rate of tumor margin angiogenesis in WIBC-9 was four times as great (or more) as that observed in MC-5, a finding consistent with the microvascular intensities observed in immunohistochemical analysis (Fig. 4) and our previously reported data, including results of immunohistochemical analysis using murine flt-1, murine tie-2, murine integrin α, β3 and murine CD31 (1).

**Morphology in the Central and Marginal Area of WIBC-9 and MC-5.** In the central area of the WIBC-9 tumor, analysis by H&E, Giemsa staining, electronmicroscopy, and immunohistochemistry with murine CD31 revealed positivity for murine CD31 and blood pooling without an EC lining (Fig. 4, A–D). These features indicated that VM, which is associated with the absence of ECs, central necrosis, and central fibrosis of IBC, xenografted the tumors. Transmission electronmicroscopy clearly visualized the tumor cell-erythrocyte interface, which did not include EC structures (Fig. 4C; T indicates tumor cells). In contrast, H&E and Giemsa staining of MC-5 revealed a medullary growth pattern, central necrosis, rare neovasculars with ECs, and positivity for murine CD31 and infiltration by mononuclear cells (Fig. 4, H–K). These features are consistent with the data obtained by MRA (Figs. 1–3).

In the marginal area and the overlying skin (Fig. 4F) of WIBC-9, macroscopic examination (Fig. 4E) and immunohistochemistry with murine CD31 (Fig. 4G) revealed hypervascularity with a lining of ECs (angiogenesis). However, the central area of WIBC-9 rarely exhibited angiogenesis, a finding consistent with the lack of a CD31 PCR band (Fig. 5B) and post-LCM observation of VM (Fig. 5A). MC-5 exhibited less angiogenesis in the marginal area of the tumor (Fig. 4N) and the overlying skin (Fig. 4M) than did WIBC-9 and exhibited central tumor necrosis (Fig. 4L, *). This strongly suggests that VM of WIBC-9 involves hemodynamics that serve to feed WIBC-9 cells, and this in turn suggests a connection between VM and angiogenesis.

**RT-PCR after LCM.** LCM was performed in the regions of VM (Fig. 5, A and D) in WIBC-9 tumors (Fig. 5A). The regions examined did not contain stromal cells or lymphocytes (Fig. 5, B and E). The HUVEC cell line was used as the positive control, and interspecies primers (human-mouse) of angiogenic factors were used. All of the primers used were for EC-associated genes. For comparison of post-LCM RT-PCR
results (Fig. 5B), we used post-LCM regions of WIBC-9 tumors (Fig. 5, C and F) and several other breast cancer cell lines, including SK-BR3 and MCF-7. Gene expression of VEGF, Flt-1, Ang-1, Ang-2, and Tie-2 was observed in samples exhibiting VM. This finding is consistent with those of our previous study, in which we used species-specific primers (1). Of particular interest is the gene expression of Flt-1 and Tie-2 in the absence of CD31, thrombin receptor, and endothelin B receptor. In addition, post-LCM regions of WIBC-9 tumors and whole tumors of WIBC-9, MC-5, MC-2, and MC-18, all of which included ECs, showed expression of all of the genes assayed for. These facts suggest that VM of WIBC-9
without ECs has a vasculogenic phenotype (Flt-1 and Tie-2), and this, in turn, suggests that VM occurs in the central area of WIBC-9 and feeds WIBC-9 cells.

**DISCUSSION**

The ubiquity of the association of angiogenesis with tumors indicates the extent to which tumor development and metastasis are dependent on neovascularization, and suggests that this relationship might involve angiogenic growth factors that are specific to neoplasms (9–22). We and others have proposed that, in certain circumstances, tumors could feed themselves without neovasculars (e.g., exploration of preexisting vessels or VM), suggesting the potential for resistance to antiangiogenic treatment (1–7, 23). Previously, it was considered impossible to prove a connection between VM and angiogenesis using angiography and the hemodynamics of VM, because there were no intravascular macromolecular MRI contrast agents that did not exhibit significant leakage through vascular walls and because there were no transplantable animal models that exhibited VM. The great advantage of the newly developed contrast agent used in the

**Fig. 5. RT-PCR after LCM.** In A, LCM was performed in the regions of VM (a and d) in WIBC-9 tumors. The regions examined did not contain stromal cells or lymphocytes (b and e). For comparison of results of post-LCM RT-PCR (B), we used post-LCM regions in WIBC-9 tumors (c and f) and post-LCM regions of MC-5 cancer cells and other breast cancer cell lines, including Sk-BR3 and MCF-7. Gene expression of VEGF, Flt-1, Ang-1, Ang-2, and Tie-2 was observed in samples exhibiting VM. Gene expression of Flt-1 and Tie-2 in the absence of CD31, thrombin receptor, and endothelin B receptor was observed. Post-LCM regions of WIBC-9 tumors and whole tumors of WIBC-9, MC-5, MC-2, and MC-18 exhibited expression of these genes.
present study, compared with conventional agents, is that leakage through vascular walls is greatly reduced (8). To the best of our knowledge, the present results, obtained from angiography and examination of the hemodynamics of VM, represent the first confirmation of a connection between VM and angiogenesis. The established xenograft WIBC-9 preserves histological and biological characteristics of human IBC. Erythema in the overlying skin, marked lymphatic permeation, and a high rate of metastasis are commonly seen in both WIBC-9 and human IBC (1, 24–26). WIBC-9 has two unique histological features: blood pooling without a lining of ECs, and tube-like structures and loops in the central tumor nests (1). Electron microscopy revealed that the tumor cell-erythrocyte interface lacks necrosis and fibrosis. These tubules, produced by VM, are lined externally with tumor cells, and no ECs are found in them.

As shown in Fig. 3, examination of the hemodynamics of VM revealed blood flow with two peaks of intensity and a statistically significant time lag, relative to the hemodynamics of angiogenesis. The observed gene expression may be the cause of the observed endothelial-vascular phenotype and the putative de novo formation of vascular channels by tumor cells. We are presently conducting experiments designed to further clarify the issues addressed by this article.

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Hemodynamics in Vasculogenic Mimicry and Angiogenesis of Inflammatory Breast Cancer Xenograft

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