Absence of Endothelial Cells, Central Necrosis, and Fibrosis Are Associated with Aggressive Inflammatory Breast Cancer

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Abstract

We recently established a new human inflammatory breast cancer (IBC) xenograft (WIBC-9) originating from a patient with IBC. The graft was transplantable in BALB/c nude and severe combined immunodeficient (SCID) mice. WIBC-9 was frequently accompanied by lung metastasis and exhibited erythema of the overlying skin, reflecting its human counterpart. Histological study of the original tumor and WIBC-9 revealed invasive ductal carcinoma with a hypervascular structure of solid nests and marked lymphatic permeation in the overlying dermis. In the central part of the solid nests, absence of endothelial cells, central necrosis, and fibrosis were observed. In vitro, WIBC-9 formed tube-like structures and loops, reflecting its in vivo feature and its human counterpart. WIBC-9 exhibited aneuploidy, ErbB-2 gene amplification, and an absence of estrogen receptor and progesterone receptor, which is consistent with IBC. Comparative studies of WIBC-9, three established non-IBC xenografts, and a human breast cancer cell line (SK-BR3) by reverse transcription-PCR, ELISA, and immunohistochemistry indicated that certain human genes (interleukin 8, vascular epidermal growth factor, basic fibrin-blast growth factor, angiopeitcin 13, Flt-1, Tie-2, and Tie-1) and certain murine genes (integrin α, β, β1, tie-2, vascular epidermal growth factor, and CD31) were overexpressed in exposure to tumor cells. The molecular basis and these unique histological features may be associated with aggressive IBC on angiogenic and nonangiogenic pathways.

Materials and Methods

Human Case Retrieval. Informed consent from each patient and the approval of the Japanese Foundation for Cancer Research Committee were obtained before each study. WIBC-9 was obtained from a 50-year-old Japanese female who presented with IBC. Histological examination of the resected specimen revealed a high-grade invasive ductal carcinoma with a solid nest pattern exhibiting marked tumor cell emboli in dilated dermal lymphatic vessels. The findings were consistent with a diagnosis of IBC (Fig. 1A). Immunohistochemically, ER and PgR were absent.

Establishment of WIBC-9. The animal protocols for all experiments were approved by the Animal Use Committee of the National Cancer Center. Tumor specimens from the patients with IBC were obtained immediately after surgery and processed as reported previously (11). Five mice were treated in each group. Tumor volume \(\left(\text{length}^2 \times \text{length}\right)\) was measured every week after tumor transplantation. All mice were sacrificed 8 weeks after tumor transplantation, when tumor cell growth had reached the exponential phase. Blood was drawn and the serum was stored at \(-80^\circ\text{C}\). The transplanted tumors and the lungs were resected. The tumor xenografts were subsequently transplanted for more than 3 years with up to 15 transplants in the same procedure, and a stable serial transplantable xenograft (WIBC-9) was successfully established in both nude and SCID mice (CLEA Japan, Tokyo, Japan).

Comparative Studies. Studies of tumorigenesis, growth rate, patterns of metastasis, and histopathology were conducted on WIBC-9. The results were compared with those of the established non-IBC xenografts including MC-2, MC-5, MC-18, and cancer cell line SK-BR3 (American Type Culture Collection, Manassas, VA; Ref. 11).

Morphological and Chromosomal Analysis. H&E, PAS, Giemsa staining of paraffin-embedded specimens and electron microscopic examinations were performed following a conventional method. For karyotype studies of the xenograft, the Giemsa G banding method was performed after 6 and 12 passages. ER and PgR were assayed by ELISA with ER-EIA and PgR-EIA kits (Dinabot, Tokyo, Japan).

In Vitro Cell Growth. The resected xenografts were passed through 200-μm gauge stainless steel mesh three times after being minced. The cells were resuspended in a medium containing 20–60% Percoll (Amerham Pharmacia Biotech, Uppsala, Sweden) and centrifuged at 1500 rpm for 20 min at room temperature. The cell pellet was collected, and erythrocytes were removed by treatment with 0.83% ammonium chloride in 10 mM Tris-HCl (pH 7.5). The remaining 1 × 10^6 cells were cultured on a type 1 collagen-coated dish (Asahi Techno Glass, Tokyo, Japan). A total of five dishes were used for each xenograft.

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2 To whom requests for reprints should be addressed, at Pharmacology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-Ku, Tokyo 104-0045, Japan. Phone: 81-3-3542-2511, extension 4450; Fax: 81-3-3542-1886; E-mail: hwakasugi@gan.ncc.go.jp.

3 The abbreviations used are: IBC, inflammatory breast cancer; SCID, severe combined immunodeficient; EGFR, epidermal growth factor receptor; ER, estrogen receptor; PgR, progesterone receptor; IL, interleukin; VEGF, vascular epidermal growth factor; bFGF, basic fibroblast growth factor; ANG, angiopoietin; RT-PCR, reverse transcription-PCR; EC, endothelial cell; PAS, periodic acid-Schiff; Ab, antibody; TGF, transforming growth factor.
Semiquantitative RT-PCR Analysis. Total RNA of each xenograft and cell line was extracted using the guanidinium thiocyanate-phenol-chloroform extraction method. The oligonucleotide primers for RT-PCR were designed to amplify specific mRNAs using published sequences. After 20, 25, or 30 cycles, the products were stained with ethidium bromide, and the relative expression levels were calculated as the density of the products divided by that of each positive control (2, 0.3, 0.3% 1, 1.0, 11$1.0$).

Flow Cytometric Analysis. Each sample was stained with the primary Abs, anti-EGFR monoclonal Ab (Oncogene Science, Uniondale, NY), anti-ErbB-2/3/4 monoclonal Ab (NeoMarker, Freemont, CA). FITC-antimouse Ab (Becton Dickinson, San Jose, CA) was then applied as the secondary Ab. The amount of each ErbB receptor was analyzed using a FACSCalibur with CellQuest software (Becton Dickinson, Mountain View, CA).

ELISA. The concentrations of human IL-1β, human IL-8, human bFGF (PharMingen), human VEGF (Immuo-Biological Laboratories Co., LTD., Fujioka, Japan), and murine VEGF (R&D Systems, Inc., Minneapolis, MN) were measured with immunoassay kits. A 100-μl aliquot of murine serum was obtained from each BALB/c nude mouse that had received a xenograft 8 weeks earlier and prepared for ELISA. A $1 \times 10^6$ cell/ml sample of each of the tumor cells was maintained in McCoy’s 5a medium containing 10% fetal bovine serum and 5 mM glutamine. After 7 days, a 100-μl aliquot of each culture supernatant was used for in vitro ELISA. Each assay was performed with triplicate wells.

Immunohistochemistry. Frozen sections of the resected tumor samples embedded in OCT Compound (Mile’s Sankyo, Tokyo, Japan) were assayed with the immunoperoxidase procedure. Antimouse CD31, antimouse integrin αβ3, anti-human integrin αβ3, antimouse flk-1, antimouse tie-2, and antihuman E-cadherin (PharMingen) were used as the primary Abs. The reaction was visualized using streptavidin-biotin (PharMingen) techniques.

Fig. 1. Morphological and chromosomal analysis. Histological features of the human original tumor revealed invasive ductal carcinoma with a solid nest pattern and blood pooling without lining of endothelia (A). Characteristic appearance of the tumor at the site of s.c. inoculation of the tumor. An anesthetized 7-week-old BALB/c nude mouse was photographed 2 weeks after s.c. inoculation on the left mammary pad and exhibits a tumor with erythema (B). Microscopic findings of WIBC-9 stained with H&E and Giemsa revealed hypervascularity, blood pooling without a lining of endothelia, and no central necrosis or fibrosis in the center of the tumor nest (C–E). Transmission electron microscopy revealed the tumor cell/erythrocyte interface (F). Phase-contrast electron microscopy clearly visualized erythrocytes between tumor cells. Erythrocytes appeared green (G). Mice with WIBC-9 (1.5 cm or more in diameter) frequently exhibited foci of metastasis in their lungs and tumor cell leakage from preexisting vessels (arrowhead, H). Karyotype analysis of WIBC-9 revealing aneuploidy and marked chromosomal abnormalities (I).
**Results**

**Establishment of WIBC-9.** The surgically resected tumors from 10 patients with IBC (Fig. 1A) were transplanted into BALB/c nude mice and SCID mice. The tumor from the ninth patient, referred to as WIBC-9, induced erythema in the overlying skin (Fig. 1B), thus showing the features of IBC. All but this case showed regression of the transplanted human breast tumors and the establishment of murine lymphomas, a result that has been reported previously (7). Histologically, WIBC-9 grew locally in an expansive manner, forming a solid nest structure and exhibiting marked lymphatic permeation. In the center of the solid nests, the tumor exhibited a lack of endothelial formation and never exhibited central necrosis (Fig. 1, C–E). This closely matched the histological features of the original tumor. Transmission and phase-contrast electron microscopy clearly visualized blood pooling without a lining of ECs in the center of the tumor nests (Fig. 1, F and G). There was no vascular structure between tumor cells and erythrocytes. Neither necrosis nor fibrosis was observed in the tumor nest. Mice with WIBC-9 (1.5 cm or more in diameter) frequently developed metastatic foci in their lungs, and tumor cell leakage from preexisting vessels was also observed (Fig. 1H). This phenotype remained stable for over 15 transplant generations. A karyotype analysis revealed chromosomal abnormalities in terms of structure and number. The median chromosome number was 75 (range, 72–77), and there was aneuploidy (n = 20; Fig. 1J).

**In Vivo and In Vitro Growth.** Once established, WIBC-9 manifested 100% tumorigenicity, with a latency of 2 weeks, and grew rapidly (Y = 46.10 × 4.14e−2X R2 = 0.960; Y, tumor volume; X, days). There was no significant difference in growth rate between WIBC-9 and MC-5. On a DNA histogram, analyzed by flow cytometer, the percentage of the S phase of WIBC-9 was 15% or greater, but this was not significant compared with those of the three other xenografts or SK-BR3.4

**Semiquantitative RT-PCR.** All ErbB receptors’ mRNAs were detected in WIBC-9 cells. The expression of ErbB-2 was detected only in WIBC-9 and SK-BR3, which is consistent with gene amplification.4 Angiogenic factors human acidic fibroblast growth factor and human TGF-α were detected only in WIBC-9, and higher levels of expression of human bFGF, human VEGF, and human ANG-1 were detected in WIBC-9 than in the explored cells. Human VEGF-C and human VEGF-D were not detected in WIBC-9. The VEGF family receptor murine flt-1 was expressed at a higher level in WIBC-9, but murine flk-1 and murine flt-4 were not detected in WIBC-9. Expression of human Flt-1 and KDR was detected in all xenografts. The human ANG receptors Tie-1 and Tie-2 were detected only in WIBC-9, and a higher level of expression of murine tie-2 was detected in WIBC-9. The cytokine human IL-1β was detected only in WIBC-9, and a higher expression of human IL-8 was detected in WIBC-9. The adhesion molecules human integrin β3, murine integrin β3, and murine integrin αv were detected at higher levels in WIBC-9. The intercellular adhesion molecule human E-cadherin was detected at the same level in all explored cells, and murine VE-cadherin was not detected in WIBC-9 (Table 1).

**Flow Cytometric Analysis.** The protein expression level of ErbB-2 was highest in WIBC-9, and WIBC-9 exhibited a high level of mean fluorescence intensity for all ErbB family receptors (Fig. 2A).

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4 Unpublished data.

**ELISA.** The protein expression level of human IL-8 was significantly higher in WIBC-9 than in MC-5, in both murine sera and culture media (Fig. 2B). Protein expression level of human bFGF and human VEGF was significantly higher in WIBC-9 than in MC-5, in both murine sera and culture media (Fig. 2, C and D). In WIBC-9, murine VEGF concentration was 3250 ± 240 pg/ml, a 31–39-fold amplification of expression in murine serum in response to the xenografting (Fig. 2E).

**Immunohistochemistry on the Tumor Margin.** Protein expression of murine flt-1 was detected in the endothelia and migrated cells of both WIBC-9 and MC-5, whereas murine flk-1 was not detected in WIBC-9 at the tumor margin (Fig. 3). The expression levels of murine tie-2 and murine integrin αβ, were 5.1–7.3-fold higher in WIBC-9 than in MC-5, and this result was consistent with the results of the semiquantitative RT-PCR examination. With regard to microvascular density, WIBC-9 exhibited significantly more intense immunoreactivity than MC-5 to murine CD31 in the neovascular epithelia of the tumor marginal area.

**Tube-like Structure in the Central Tumor in Vitro and in Vivo.** In vitro, the prepared WIBC-9 cells formed tube-like structures and loops consisting of solid cords in the basement membrane matrix (Fig. 4A). The in vitro features were similar to those observed in the IBC specimens containing erythrocytes (Fig. 4B). Note that these tubes are lined externally with tumor cells, and no ECs are identified.

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**Table 1** Semiquantitative RT-PCR of WIBC-9 and non-IBC xenografts

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<th>MC-5</th>
<th>SK-BR3</th>
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*Expression of genes for epidermal growth factors, angiogenic factors, cytokines, and EC adhesion molecules was assayed with RT-PCR. The relative expression levels were calculated as the density of the products divided by that of each internal control (−0.3, 0.3 ≤ ± < 1.0, ++ ± ≥ 1.0). M-CSF, macrophage colony-stimulating factor.*
Transmission electron microscopy visualized the tube-like structures and loops containing erythrocytes of WIBC-9 in vivo (Fig. 4, c and d). WIBC-9 exhibited PAS-positive components of loops and blood pooling (Fig. 4e). Weak protein expression of human integrin $\alpha_v\beta_3$ was detected in the tube-like structures (Fig. 4f).

**Discussion**

The established xenograft WIBC-9 preserves histological and biological characteristics of human IBC. The features of erythema in the overlying skin, marked lymphatic permeation, and high rate of metastasis are commonly seen in both WIBC-9 and human IBC. In this respect, WIBC-9 is the second IBC xenograft model. WIBC-9, however, has two unique histological features: (a) blood pooling without a lining of ECs and (b) partially with the tube-like structures and loops in the central tumor nests. Electron microscopy visualized the tumor cell/erythrocyte interface lacking necrosis or fibrosis. These structures exhibited weak expression of the human activated endothelial marker, human integrin $\alpha_v\beta_3$, and were PAS positive. These tubules are lined externally with tumor cells, and no ECs were identified. These results suggest de novo formation of vascular channels by tumor cells in the central area of the xenografted tumor in the regimes of hypoxia and up-regulated angiogenic factors. They also suggest that vessel regression has not been occurring in these tumors and that the blood from ruptured vessels has not filled tumor-lined lakes or channels. WIBC-9 exhibited the absence of fibrosis, central necrosis, or lining of endothelia, whereas MC-2, MC-5, and MC-18 commonly exhibit fibrosis and central necrosis as the tumor grows. We believe that these findings may be related to the expression of certain genes in WIBC-9 (i.e., human Flt-1, human Tie-2, human Tie-1, and human integrin $\alpha_v\beta_3$). This gene expression may result in the observed endothelial/vascular phenotype and the putative de novo formation of the vascular channel by tumor cells. In the tumor margin, WIBC-9 exhibited hypervascularity and significantly more intense immunoreactivity of murine CD31 in the neovascular epithelia than non-IBC xenografts. This may explain endothelial sprouting of new vessels from preexisting vessels as a result of overexpression of the angiogenic factors.

Inflammatory cytokines, including IL-1, IL-8, and TNF-$\alpha$, are secreted from macrophages, neutrophils, lymphocytes, ECs, and tumor cells. They are involved in migration and inactivation of inflam-
It has been speculated that inflammatory cytokines may modulate angiogenesis (12). Inflammatory cytokines not only exert direct action on ECs but also exert indirect action on migrating macrophages and lymphocytes that secrete other cytokines (13, 14). They directly affect angiogenesis and indirectly modulate adhesion molecules and matrix metalloproteinase. IL-8, which is a member of the ELR+ CXC chemokine families, has been reported to be an EC migration accelerator and a bFGF accelerator (15).

ANG-1 is a strong inducer of EC sprouting, which is a first step in both angiogenesis and neovascularization (16). VEGF and bFGF stimulate EC sprouting, and the stimulated ECs, in turn, induce the production of ETS-1, which up-regulates angiogenesis by promoting the activation of protease and integrin β3 (17). In WIBC-9, human angiogenic factors (ANG-1, VEGF, and bFGF) and murine angiogenic factors (flt-1, integrin β3, VEGF, and CD31), were expressed at higher levels than they were in the three non-IBC xenografts. On the host side, murine VEGF exhibited a 31–39-fold amplification of expression in WIBC-9 in response to xenografted cells. Human VEGF-C and human VEGF-D have been postulated to be molecules characteristic of IBC because Flt-4, which is known to be one of the receptors of VEGF-C and VEGF-D, is present in the epithelium of the lymphatic ducts. However, human VEGF-C, human VEGF-D, and murine flt-4 were not detected in WIBC-9.

Integrin αβ3, which is the most well-documented integrin family, introduces angiogenesis in the endothelium. Blocking of integrin αβ3 results in the regression of angiogenesis and induces apoptosis of ECs (18). In WIBC-9, murine integrin α and murine integrin β3 were amplified by RT-PCR, and murine integrin αβ3 was immunohistochemically determined to be overexpressed. In WIBC-9, the expression levels of human angiogenic factors (VEGF, ANG-1, bFGF, and...
IL-8) on the graft side and murine angiogenic factors (integrin β3, lt-1, tie-2, VEGF, and CD31) on the host side were significantly high. This may explain why angiogenic factors from the tumor promote the production of host angiogenic factors, including the elevated integrin αvβ3 in the epithelium. Elevated host integrin αvβ3 and other factors derived from migrated host cells up-regulate murine VEGF and other host angiogenic factors paracrine among the cells, thus leading to the formation of a hypervascular tumor on the tumor margin. The activated form of Flt-1 kinase-transfected fibroblasts forms a tube-like structure in the basement membrane matrix (19). Possible similarities between the characteristics of migrated tumor cells and those of activated ECs and endothelial precursor cells are now under investigation. VE-cadherin is specific to the endothelium. When the VE-cadherin gene is knocked out, tube formation is not observed (20). In WIBC-9, this molecule was not detected. This finding may also be related to the fact that WIBC-9 lacks endothelial formation in the central tumor.

This molecular basis and these unique histological features may be associated with aggressive IBC on angiogenesis and nonangiogenesis.

Acknowledgments

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References

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