A Novel Human Xenograft Model of Inflammatory Breast Cancer

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Abstract

The step of intravasation or lymphovascular invasion can be a rate-limiting step in the metastatic process. Inflammatory breast carcinoma manifests an exaggerated degree of lymphovascular invasion in situ; hence, a study of its molecular basis might shed light on the general mechanism of lymphovascular invasion exhibited by all metastasizing cancers. To this end, we have established the first human transplantable inflammatory breast carcinoma xenograft (MARY-X) in scid/nude mice. Whereas all other human xenografts grew as isolated s.c. nodules, MARY-X grew exclusively within murine lymphatics and blood vessels, and these latter elements and their supporting stroma comprised, by murine Cot-I DNA analysis, 30% of the tumor. MARY-X, like its human counterpart, exhibited striking erythema of the overlying skin. MARY-X was estrogen receptor, progesterone receptor, Her-2/neu negative and p53, epidermal growth factor receptor positive. The primary tumor of origin of MARY-X exhibited identical markers, except that about 50% of its cells exhibited Her-2/neu amplification. Comparative studies of MARY-X with noninflammatory xenografts indicated 10–20-fold overexpression of E-cadherin and MUC1, findings that were reflected in actual cases of human inflammatory breast cancer. MARY-X should allow us to further dissect out both the upstream regulatory machinery and the downstream effector molecules responsible for the inflammatory carcinoma phenotype.

Introduction

Recent experimental studies have suggested that intravasation is a rate-limiting although poorly understood step of the metastatic process (1, 2). Inflammatory breast carcinoma is a disease of humans that exhibits an exaggerated degree of intravasation in situ manifested by florid invasion of lymphatic and vascular capillaries. Inflammatory breast cancer is one of the most aggressive types of human breast cancer (3). Clinically, patients present with an inflamed tender breast with the so called erysipelas edge and/or peau d’orange. Pathologically, there is extensive lymphovascular invasion by tumor emboli, which involve the superficial dermal plexus of vessels in the papillary dermis and high reticular dermis. Although clinically a dominant mass may or may not be present, pathologically there is often a diffuse mass of invasive breast carcinoma of high histological grade according to the Bloom-Richardson-Scarf classification (poor differentiation with high nuclear grade; Ref. 4). Along with aggressive histological features, inflammatory carcinomas often exhibit aggressive biomarkers, including high S phase (>15%), aneuploidy, negative ER3 and PR receptors, increased expression of EGFR, and Her-2/neu amplification (5). Inflammatory carcinomas tend to exhibit axillary nodal metastases, a high incidence of local and systemic recurrence, and distal metastases. Inflammatory carcinomas can occur in either primary or secondary forms, the latter term referring to a noninflammatory primary carcinoma that recurs as an inflammatory carcinoma. It is interesting that locally advanced noninflammatory primary cancers that are successfully treated with neoadjuvant chemotherapy often show evidence microscopically of residual carcinoma present almost exclusively in lymphovascular channels.

Because the “inflammatory phenotype” had not been successfully reproduced in an animal model, we decided that to dissect out the molecular basis of this phenotype we had to first establish an experimental model of inflammatory carcinoma that recapitulated the inflammatory phenotype. This study reports the establishment of a human transplantable inflammatory carcinoma xenograft (MARY-X) in scid/nude mice. Although all other human xenografts grew as isolated s.c. nodules (6), MARY-X grew exclusively within murine lymphatics and blood vessels and was the first such human xenograft to do so.

Materials and Methods

Cell, Xenograft Establishment, and Human Case Retrieval. Informed patient consent and certification from the UCLA Human Subject Protection Committee were obtained prior to all studies. Approval from the Chancellor’s Animal Research Committee was requested and obtained (certification ARC 95-127-11). The MARY-X xenograft was established directly from a 45-year-old female who presented with a warm and erythematous breast and ill-defined mass. The mass was biopsied and diagnosed as inflammatory carcinoma exhibiting florid invasion of dermal lymphatics. Minced 1-mm3 portions of the biopsy were washed in HBSS, placed in RPMI 1640, and subsequently implanted s.c. in several female scid and athymic (nude) mice (nu/nu mutants on a BALB/c background). The tumoral xenografts that grew were subsequently transplanted when they reached 1 cm in diameter. A stable serial transplantable xenograft was successfully established in both scid and nude mice. Studies of tumorigenicity, growth rate, animal-associated characteristics, patterns of metastasis, tumor histology, immunocytochemistry, and molecular characterizations were conducted on MARY-X and compared with studies on established noninflammatory xenografts including the MDA-MB-231 and MDA-MB-468 breast carcinoma xenografts and our human myoepithelial xenografts (HMS-X, HMS-3X, and HMS-4X), which we established previously (7–9). The MARY-X “shake,” an enriched population of human tumor cells, 99% free from murine components, was produced by extirpating MARY-X, placing it in culture medium, immediately making parallel incisions without transecting the tumor, and gently agitating the culture medium at 4°C for 2 min. The tumor proper was then removed, and the tumor shake was centrifuged at 1700 × g for 2 min. The shake consisted of spheroids that grew both in suspension as well as attached to monolayers. This shake was placed in suspension culture in keratinocyte serum-free medium (Life Technologies, Inc., Gaithersburg, MD) and also grown on monolayers of normal human mammary epithelial cells (Clonetics, San Diego, CA) and human umbilical vein endothelial cells (Clonetics). A murine specific Cot-I probe (see below) verified that the MARY-X shake was 99% human.

Cases of human inflammatory and noninflammatory breast carcinoma were retrieved from archival pathology material, Center for the Health Sciences, UCLA and used for selected immunocytochemical studies.

DNA Profiling. High molecular weight DNA was extracted from the xenografts and host murine tissues by standard procedures (10), digested with HaeIII and HinfI, and probed with the microtlocus 33.6 Jeffreys probe (Ref. 11; Cellmark Diagnostics, Germantown, MD). This probe recognizes both human
as well as murine DNA. A human-specific [α-32P]dCTP-labeled human Cot-1 DNA probe (Life Technologies, Inc.) was used to confirm the human identity of the xenograft. A murine-specific [α-32P]dCTP-labeled mouse Cot-1 DNA probe (Life Technologies, Inc.) was used to distinguish murine from human DNA and quantify the murine DNA component of the xenografts.

**Western Blot and Zymography Analysis.** MARY-X was compared with noninflammatory xenografts with respect to candidate effector molecules. Tissue lysates and primary antibodies to candidate adhesion molecules and angiogenic factors were used in standard Western analyses (12). Goat antimouse antibodies were used as secondary antibodies, and the signals were developed with the ECL System (Amersham Life Sciences, Arlington Heights, IL.). Standard zymography analysis was also used to compare levels of proteolytic enzymes (13).

**Immunocytochemistry and FISH.** MARY-X, its primary tumor of origin, and the noninflammatory xenografts mentioned previously were studied with selected monoclonal antibodies to ER and PR (Abbot Laboratories, Chicago, IL), EGFR (Ciba-Corning Diagnostic Corp., East Walpole, MA), and p53 and Her-2/neu (Oncogene Sciences, Inc., Uniondale, NY.). MARY-X was also studied with polyclonal rabbit antibodies to von Willebrand factor (DAKO, Carpinteria, CA) and monoclonal antibodies to E-cadherin (Zymed Laboratories, Inc., San Francisco, CA) and MUC1 (Research Diagnostics, Inc., Flanders, NJ). The latter two antibodies were also used in cases of human inflammatory and noninflammatory carcinoma. All antibodies were used at their manufacturers’ recommended dilutions. Peroxidase-conjugated sheep antimouse IgG or goat antirabbit was used as secondary antibody. Colorimetric detection of peroxidase-conjugated secondary antibody was with diaminobenzidine. Her-2/neu FISH was carried out on tissue sections of both MARY-X as well as its primary tumor of origin with a Her-2/neu probe spanning ~140 kb of the chromosomal region. This probe was directly labeled with the fluorophore SpectrumOrange (Vysis, Inc., Downers Grove, IL; Ref. 14). As a control for chromosomal 17 aneusomy, a chromosome 17-specific centromeric α-satellite probe (D17Z1; Ref. 14; Vysis, Inc.) was used. This latter probe was biotin labeled and detected via avidin-FITC. Tissue sections were counterstained using 0.15 mM 4,6-diamidino-2-phenylindole (Sigma Chemical Co., St. Louis, MO). A Zeiss epifluorescence microscope with single band pass filters was used to measure the different wavelength fluorescences.

**Results**

We report the successful establishment of the first human transplantable inflammatory breast carcinoma xenograft (MARY-X) in scid/nude mice. Remarkably, MARY-X induced erythema in the overlying mouse skin (Fig. 1A), mimicking the clinical presentation of inflammatory carcinoma. Although all other human xenografts grew as isolated s.c. nodules, MARY-X grew exclusively within murine lymphatic and blood vessel channels (Fig. 1, B–D). Confirmation of the vascular identity of these channels was on the basis of von Willebrand factor immunoreactivity. Analysis of the lungs of mice with large MARY-X tumors (1.5–2.0-cm diameter) revealed the presence of pulmonary metastases, but surprisingly, these metastases were confined to within vessels (Fig. 1E). No extravasation of these pulmonary metastases occurred. The phenotype of MARY-X was limited then to intravasation. This phenotype has remained stable in over 15 transplant generations.

DNA fingerprinting, the canonical method of proving that a cell line or xenograft is original, served also to demonstrate that MARY-X had a significant (~30%) murine component (Fig. 2, A and B), presumably attributable to murine vessels and their supporting stroma. Once established, MARY-X manifested 100% tumorigenicity with a latency of ~1 week and grew fairly rapidly (Fig. 2C). This growth, however, was confined to lymphovascular spaces. A human-specific Cot-1 DNA probe documented human DNA in MARY-X (data not shown). Using a murine- specific Cot-1 probe, the murine component of MARY-X could be quantitated and compared with the murine component of other human xenografts, and MARY-X demonstrated the greatest murine percentage (~30%; Fig. 2D), presumably from its murine lymphovascular component and supporting stroma. We were able to effectively separate the human tumor cells from the murine vascular component by producing a MARY-X shake in vitro that was 98–99% human (Fig. 2D). Subsequent studies on select gene expression of MARY-X was usually done on this MARY-X shake to exclude as a source of gene expression the murine vascular component. The MARY-X shake produced spheroids that could be maintained as suspension cultures for 3 months or that could attach to epithelial (human mammary epithelial cells) or endothelial (human umbilical vascular endothelial cell) monolayers. These spheroids remained viable for periods up to 3 months but showed evidence of hypoxia and necrosis in their centers after ~4 weeks in culture (Fig. 2E).

MARY-X, like MDA-MB-231 and MDA-MB-468, was ER, PR, Her-2/neu negative, and p53, EGFR positive (Fig. 3). Some of these markers showed homogeneously, and some, in fact, were quite heterogeneous. p53 immunostaining was present in nearly 100% of the cells; ER and PR staining was 100% absent; EGFR was strongly immunoreactive in 20% of the cells. The primary tumor of origin of MARY-X exhibited identical markers as MARY-X, with the exception of Her-2/neu. About 50% of the primary tumor of MARY-X showed marked Her-2/neu amplification by both immunocytochemical staining (Fig. 3D) and FISH (Fig. 3E). MARY-X, however, showed no evidence of Her-2/neu amplification (Fig. 3F), despite maintaining its inflammatory signature of florid lymphovascular invasion.

Because inflammatory carcinomas manifest striking lymphovascular invasion, it was reasonable to suppose that they might stimulate angiogenesis/lymphangiogenesis and then manifest lymphovascular “homing.” It was also reasonable to suppose that the molecular mechanism(s) of this effect might involve adhesion molecules, either on tumor cells or endothelial cells, or angiogenic growth factors and proteolytic enzymes elaborated by tumor cells that enable intravasation. We conducted an initial screen of candidate effector molecules implicated previously in the abovementioned processes. These molecules included candidate adhesion molecules of the integrin family, the immunoglobulin superfamily and other adhesion molecules; candidate angiogenic factors of the vascular endothelial growth factor, fibroblast growth factor, and transforming growth factor families; and candidate proteases and their receptors including serine and metalloproteinases. Of these molecules examined, two initially stood out: MUC1 and E-cadherin. MARY-X compared with noninflammatory xenografts markedly overexpressed (10–20 fold) MUC1 (Fig. 4A) and E-cadherin (Fig. 4B). These findings on Western blot were confirmed in both immunocytochemical studies of MARY-X and in actual cases of human inflammatory breast cancer. Lymphatic tumor emboli of inflammatory carcinoma manifested strong membrane E-cadherin immunoreactivity (Fig. 4C) and strong cytoplasmic and membrane MUC1 immunoreactivity (Fig. 4D) in 100% of the cases of human inflammatory carcinoma that were examined. Interestingly, increased membrane E-cadherin immunolocalization was also observed in cases of noninflammatory breast carcinoma but only in their foci of lymphovascular invasion (data not shown).

**Discussion**

Very little is known about the pathogenesis of inflammatory breast cancer. The incidence of inflammatory carcinoma varies within ethnic and socioeconomic groups, geographically and strictness of applied clinicopathological criteria. Considering all of these factors, the incidence is reported to be between 1 and 10% of all breast cancers. Interestingly, inflammatory carcinoma is a type of breast cancer...
common in Tunisia and Morocco, suggesting that either local environmental factors or hereditary factors can trigger its occurrence (15). It is also common in ethnic groups with locally advanced cancers because of poor medical care and absent screening. In any case, it can be argued that the exaggerated phenotype exhibited by inflammatory carcinoma is exhibited to a lesser degree by all breast cancers that do not remain organ confined. After all, breast cancers that metastasize must exhibit either lymphatic or vascular invasion at some point in their metastatic route. Therefore, an understanding of the molecular basis of inflammatory carcinoma might increase our understanding of the metastatic process of all breast cancers. Because inflammatory carcinomas manifest striking lymphovascular invasion, it was reasonable to suppose that they might stimulate angiogenesis/lymphangiogenesis and then manifest lymphovascular homing. It was also reasonable to suppose that the molecular mechanism of this effect might involve adhesion molecules either on tumor cells or endothelial cells or angiogenic growth factors and proteolytic enzymes elaborated by tumor cells that enable intravasation. Because exaggerated lympho-

Fig. 1. MARY-X turns the overlying murine skin bright red (A). MARY-X consists grossly of a confluence of white nodules (B) that correspond to distended lymphovascular channels filled with tumor emboli (C). These vascular channels represent lymphatics and blood vessels and demonstrate von Willebrand factor immunoreactivity, as depicted by circumferential brown staining. Higher magnification of MARY-X depicts lymphovascular invasion (D). Interestingly, MARY-X exhibits only the step of intravasation in both the primary tumor as well as in pulmonary metastases (E), but the pulmonary emboli do not extravasate and establish true pulmonary metastases, even after prolonged time periods.
vascular invasion is exhibited by only a minority of human breast cancers and because previously established human nude mouse xenografts of breast carcinoma cell lines do not exhibit lymphovascular invasion (yet express many of the known adhesion molecules, angiogenic growth factors, and proteolytic enzymes involved in angiogenesis and invasion), it was reasonable to hypothesize that the mechanism of inflammatory carcinoma might involve both upstream regulatory mechanisms and downstream effector molecules still undiscovered.

To begin to address these issues, we needed an animal model of inflammatory carcinoma, and we exploited the fact that nude and scid mouse xenografts of human tumors often recapitulate their autochthonous phenotype in their murine host (6). Our studies comparing MARY-X with the noninflammatory xenografts MDA-MB-231 and MDA-MB-468 demonstrated that the murine component is detected in MARY-X, as evidenced by a number of comigrating bands (Lanes C versus D). C, MARY-X growth kinetics in nude mice. Although MARY-X exhibited fairly rapid tumor growth, its growth was confined to progressive distension and involvement of murine vessels. Using a mouse-specific Cot-1 DNA probe (D), human tumoral xenografts HMS-X, HMS-3X, HMS-4X, MDA-MB-231-X, and MDA-MB-468-X demonstrated a variable murine component ranging from negligible to significant. MARY-X demonstrated the greatest murine component. A control human melanoma line C8161-C predictably exhibited no murine DNA component. A human tumor cell-enriched fraction of MARY-X, the MARY-X shake (E), is composed of spheroids with pale-staining central areas, evidence of hypoxia and necrosis after 4 weeks in suspension culture.
phenotype. These comparative studies indicate that none of the known markers of breast carcinoma progression, although often altered in human inflammatory carcinoma, is sufficient alone in mediating its phenotype.

Our comparative approach with MARY-X versus the noninflammatory xenografts has initially yielded two molecules that are overexpressed: MUC1 and E-cadherin. Both of these molecules were similarly overexpressed in actual cases of human inflammatory carcinoma by immunocytochemical studies. Although these preliminary studies certainly have not demonstrated that either of these molecules are functionally intact nor that they are the effector molecules that actually mediate the inflammatory phenotype, it is attractive to postulate that these molecules may contribute to the homotypic (tumor cell-tumor cell) and heterotypic (tumor cell-endothelial cell) emboli so characteristic of inflammatory carcinoma and so in keeping with their known functions (16–18).

With Mary-X, there are a number of other comparative molecular approaches that are available to us including differential display, cDNA expression microarray analysis, phage display, and proteomics which we anticipate using. All these approaches are designed to ask, “what is different about the inflammatory phenotype?” This question is the most important question of all.

Fig. 3. MARY-X was p53 positive (A), ER negative (B), PR negative, EGFR positive (C), and HER-2/neu negative. The primary tumor of origin of MARY-X, however, showed Her-2/neu amplification by immunocytochemistry (D) and FISH (E) in a significant fraction of its cells. With FISH, strong yellow-orange fluorescence (fluorophore SpectrumOrange) is depicted in the cell population at the lower portion of the slide (E). Cell population at the upper portion shows unamplified Her-2/neu. A chromosome 17-specific centromeric α-satellite probe (D17Z1) revealed normal ploidy in all of these areas. FISH reveals that MARY-X has completely lost the Her-2/neu amplified population (F).
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References

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