Cortactin Potentiates Bone Metastasis of Breast Cancer Cells

Yansong Li, Mehrdad Tondravi, Jiali Liu, Elizabeth Smith, Christian C. Haudenschild, Michele Kaczmarek, and Xi Zhan

Departments of Experimental Pathology [Y. L., J. L., E. S., C. C. H., X. Z.] and Hematopoiesis [M. T., M. K.], Holland Laboratory, American Red Cross, Rockville, Maryland 20855, and Department of Anatomy and Cell Biology, The George Washington University, Washington, D.C. 20037 [X. Z.]

ABSTRACT

Gene amplification of the chromosome 11q13 in breast cancer and squamous carcinomas in the head and neck results in frequent overexpression of cortactin, a prominent substrate of Src-related tyrosine kinases in the cell cortical areas. To investigate the role of cortactin in tumor progression, we analyzed MDA-MB-231 breast cancer cells overexpressing green fluorescent protein-tagged murine cortactin (GFP-cortactin) and a cortactin mutant deficient in tyrosine phosphorylation under the control of a retroviral vector. Injection of MDA-MB-231 cells overexpressing GFP-cortactin into nude mice through cardiae ventricles caused bone osteolysis at a frequency ~85% higher than that of cells expressing the vector alone, whereas injection of cells overexpressing the mutant deficient in tyrosine phosphorylation induced 74% fewer osteolytic metastases as compared with the control group. Interestingly, the cells expressing either GFP-cortactin or the mutant did not show significant differences in growth in vitro or when injected m.f.p. in vivo. On the other hand, the cells overexpressing GFP-cortactin but not the mutant acquired a >60% enhanced capability for transendothelial invasion and endothelial cell adhesion. These data suggest that cortactin contributes to tumor metastasis by enhancing the interaction of tumor cells with endothelial cells and the invasion of tumor cells into bone tissues.

INTRODUCTION

Breast cancer cells are known to spread preferentially to bone tissues and ultimately develop osteolytic metastasis (1, 2). Bone metastasis is a major cause of the decline in the quality of life of patients because of uncontrollable bone pain, pathological fractures, hypercalcemia, and nerve compression syndromes (3). Despite these well-recognized clinical syndromes, the mechanism that causes breast tumors to target bone tissue remains unclear. Breast cancer is frequently associated with gene amplification at chromosome 11q13, resulting in overexpression of cortactin (EMSL1; Ref. 4), a cortical actin-association protein that is a prominent substrate of the protein tyrosine kinase Src (5, 6). Gene amplification of cortactin is also found frequently in other types of cancer including head and neck squamous carcinoma and bladder cancer (7, 8). Whereas the precise role of cortactin in tumor progression remains unclear, amplification and overexpression of cortactin appear to be intimately associated with patients with poor prognosis or relapse (9). This indicates that overexpression of cortactin may contribute to a late stage of tumor progression.

Cortactin is accumulated in peripheral structures of cells, including lamellipodia and membrane ruffles, where cortical actin is enriched (10). In MDA-MB-231 breast cancer cells plated on extracellular matrix, cortactin is enriched in invadopodia, a type of membrane protrusion that participates in degradation of and invasion into the matrix (11). The protein sequence of cortactin features six and a half tandem copies of a unique 37-amino acid repeat domain and a SH3 domain at the COOH terminus. Our previous studies have determined that Src-mediated tyrosine phosphorylation occurs primarily at residues Tyr-421, Tyr-466, and Tyr-482, which lie between the repeat and SH3 domains. In vitro, cortactin binds to and cross-links F-actin into meshwork. The F-actin cross-linking activity of cortactin can be reduced on tyrosine phosphorylation mediated by Src (5). Inhibition of tyrosine phosphorylation of cortactin by a selective Src inhibitor reduces the response of endothelial cells to hydrogen peroxide-mediated cell injury (12). Likewise, overexpression of a cortactin mutant that is deficient in tyrosine phosphorylation can compromise cell shape changes induced by reactive oxygen species, whereas overexpression of wild-type cortactin results in enhancement of the injury response to hydrogen peroxide (12). The role of cortactin in cytoskeletal reorganization is further highlighted by the recent finding that cortactin binds to the Arp2/3 complex and activates Arp2/3 complex-mediated actin polymerization (13). Thus, cortactin appears to act as a signaling molecule in the regulation of the dynamics of actin cytoskeleton in a tyrosine phosphorylation-dependent manner.

Although the biochemical and cellular function of cortactin and its relationship to poor prognosis in a subset of cancers suggest that cortactin may play a role in tumor metastasis, direct evidence is lacking. In this study, we examined the metastatic ability of MDA-MB-231 breast cancer cells overexpressing wild-type cortactin and a cortactin mutant deficient in tyrosine phosphorylation. We report here that overexpression of wild-type cortactin promoted the metastatic potential of tumor cells, whereas overexpression of the phosphorylation-deficient cortactin mutant inhibited metastasis. In addition, we demonstrate that cortactin influences the interaction of tumor cells with endothelial cells. Thus, our study provides, for the first time, direct evidence of the role of cortactin in tumor metastasis.

MATERIALS AND METHODS

Reagents. Unless stated otherwise, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). LipofectAMINE and G418 were purchased from Life Technologies, Inc. (Rockville, MD). Protein A-Sepharose CL-4B and enhanced chemiluminescence Western blotting kits were from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Monoclonal anti-phosphotyrosine antibody (4G10) and monoclonal anti-cortaktin antibody (4F111) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal cortactin antibody was prepared as described previously (14).

Cell Culture. MDA-MB-231 cells were grown in DMEM supplemented with 10% (v/v) FBS. Human BMECs (a gift from Malcolm Moore; Memorial Sloan-Kettering Cancer Center, New York, NY) were maintained in Iscove’s modified Dulbecco’s medium (Life Technologies, Inc.) supplemented with 20% FBS, 1× antibiotic-antimycotic solution, and 2 mM l-glutamine in a 75-cm 2 tissue culture flask (Corning).

Construction and Preparation of Cortactin Virus. Retroviruses encoding GFP-cortactin and GFP-Cort F421F466F482 were constructed and prepared as described previously (12). The MGIN viral vector was a gift of Robert Hayler.

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2 To whom requests for reprints should be addressed, at Holland Laboratory, American Red Cross, 15601 Crabbs Branch Way, Rockville, MD 20855. Phone: (301) 738-0568; Fax: (301) 517-0352; E-mail: zhanx@usa.redcross.org.

3 The abbreviations used are: SH3, Src homology 3; FBS, fetal bovine serum; GFP, green fluorescence protein; GFP-cortactin, green fluorescence protein-tagged cortactin; BMEC, bone marrow endothelial cell; FACS, fluorescence-activated cell sorting.
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(American Red Cross Holland Laboratory, Rockville, MD), and has been described previously (15).

Viral Infection. MDA-MB-231 cells were plated on 35-mm dishes at a density of 1 × 10^5 cells/dish. On the next day, the medium was replaced with 1 ml of viral supernatant containing 8 μg/ml Polybrene. After 48 h of incubation, the culture medium was replaced with DMEM containing 10% FBS. Expression of GFP proteins was monitored by fluorescence microscopy.

To increase the efficiency of infection, the cells were reinoculated with the virus two or three times, and the infected cells were enriched further by FACS.

FACS Analysis. MDA-MB-231 cells (2 × 10^6) infected with cortactin viruses were trypsinized, washed, and suspended in PBS supplemented with 2% FBS. The suspended cells were sorted in a FACS system (Becton Dickinson, Franklin Lakes, NJ) according to light scatter and fluorescence intensity.

Sorted cells with expression efficiencies from 85–98% were used for further analysis.

Phosphotyrosine Immunoblot Analysis. Cells were extracted in lysis buffer [50 mM Tris-HCl (pH 7.4) containing 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 2 mM Na_3VO_4, and 1 mM NaF]. The extracts were centrifuged at 14,000 rpm for 10 min at 4°C. The clarified supernatants were immunoprecipitated with 5 μg of polyclonal cortactin antisera (14). The immunoprecipitates were resolved by SDS-PAGE (7.5%, w/v), transferred to a nitrocellulose membrane, and further blotted with a monoclonal phosphotyrosine antibody (4G10). To measure the expression levels of cortactin, the blot membrane was stripped and reblotted with monoclonal cortactin antibody (4F11).

Cell Growth Assay. MDA-MB-231 cells were seeded on day 0 in a 12-well plate at a density of 0.4 × 10^5 cells/well in DMEM supplemented with 10% FBS and 1 × antibiotic-antimycotic solution (Life Technologies, Inc.). At various times from days 1–6, cells were trypsinized and counted with a hemocytometer under a phase-contrast light microscope. Quadrupled samples were analyzed for each time point.

Colony Formation Assay. Cells were trypsinized and resuspended in DMEM plus 10% FBS. The suspended cells (500) were mixed with 1 ml of 0.4% top agarose (SeaPlaque; FMC BioProducts, Rockland, ME) in DMEM plus 10% FBS. The mixture was plated onto a 35-mm Petri dish containing 1 ml of 0.6% bottom agarose in the same culture medium and incubated at 37°C, 5% CO_2. After 2 weeks, colonies were examined under a fluorescence microscope. The colonies with diameters of >100 μm were counted and averaged based on the 18 images. For each cell line, four independent dishes were examined.

Tumorigenicity of MDA-MB-231 Cells in the Mammary Fat Pad of Nude Mice. All animal studies described here were performed according to protocols approved by the Institutional Animal Care and Use Committee of Holland Laboratory. Tumorigenicity of MDA-231 cells was determined based on a modified method (16). Briefly, cells (2 × 10^6) were suspended in 0.2 ml of 50% (v/v) Matrigel (Collaborative Research, Bedford, MA) in PBS. Four-week-old female nude mice were anesthetized with ketamine (30 μg/g) and xylazine (1.5 μg/g). The mammary fat pad of a mouse was exposed by a skin incision in the right lateral thorax, and the cells were inoculated into the tissue using a 23-gauge needle. For each cell sample, eight mice were analyzed. Four weeks after injection, the animals were sacrificed, and the tumors were removed and weighed.

Analysis of Adhesion of MDA-MB-231 Cells to Human BMECs. The procedure was based on a modified method, as described in Ref. 17. Briefly, human BMECs were plated on fibronectin-coated 2-well Lab-Tek chamber slides (Nunc, Inc., Naperville, IL) at a density of 2 × 10^5 cells/well. After cells were confluent, MDA-MB-231 cells expressing GFP-cortactin variants were trypsinized, resuspended in DMEM containing 0.1% BSA and 1 mM CaCl_2, and plated over monolayers of endothelial cells that had been washed twice with PBS immediately before plating. The cells were incubated at 37°C for 4 h in a CO_2 incubator. Nonattached cells were removed by three washings with PBS. Attached cells were fixed with 3.7% formaldehyde for 30 min. Cells that adhered to endothelial cells were inspected under a fluorescence microscope equipped with a digital camera and quantified by counting the number of green cells based on five high-power field digital images taken randomly at ×200. The average number of adherent cells and the SD were calculated based on three independent experiments.

Analysis of Transendothelial Invasion by Tumor Cells. Transendothelial invasion of MDA-MB-231 cells was analyzed based on a modified method, as described in Ref. 18. Briefly, human BMECs (3 × 10^5) were plated on a fibronectin-coated polycarbonate membrane insert (6.5 mm in diameter with 8.0-μm pores) in a Transwell apparatus (Costar, Cambridge, MA) and maintained in Iscove’s modified Dulbecco’s medium containing 20% FBS, 1 × antibiotic-antimycotic solution, and 2 mM L-glutamine. After cells reached confluence, MDA-MB-231 cells expressing GFP-cortactin variants were trypsinized and resuspended in DMEM containing 10% FBS. The suspended cells (3 × 10^5) were seeded on the monolayer of endothelial cells and incubated for 20 h at 37°C in a CO_2 incubator. After incubation, the insert was washed with PBS. The cells on the top surface of the insert were removed by wiping with a cotton swab. The cells that migrated to the bottom surface of the insert were fixed with 3.7% formaldehyde and subjected to fluorescence microscopic inspection. Green cells were counted based on five high-power field digital images taken randomly at ×200. The average number cell number and SD were calculated based on duplicated experiments.

Intracardiac Injections of MDA-MB-231 Cells in Nude Mice. Subconfluent MDA-MB-231 cells were fed with DMEM containing 10% FBS 24 h before injection. The cells were trypsinized, immediately suspended in DMEM containing 0.2 mg/ml soybean trypsin inhibitor, and washed twice with PBS. The washed cells were finally resuspended in cold PBS at a density of 2.5 × 10^6 cells/ml on ice. Female 4–5-week-old BALB/c-nu/nu mice (National Cancer Institute, Frederick, MD) were anesthetized with ketamine (30 μg/g) and xylazine (1.5 μg/g). The suspended cells (5 × 10^6) were injected on April 10, 2017. © 2001 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from
into the left cardiac ventricles of animals with a 27-gauge needle. The cell-injected animals were housed in a pathogen-free environment for 5–10 weeks. Body weights of animals were measured using a digital Sartorius weigher (The Scale People, Inc., Beltsville, MD). Experiments were repeated twice, and each experiment involved five animals per cell sample.

**Statistical Analysis.** Statistical evaluation of the differences among groups was performed by Mann-Whitney test using GraphPad InStat software. All data shown were the mean ± SD.

**Determination of Bone Metastases by X-radiography.** Tumors in bone were examined by X-ray radiographs 5 weeks after injection. Animals were anesthetized and placed on a transparent board in prone and lateral positions. The board was placed against an X-ray film (22 × 27 mm; X-OMAT AR; Kodak, Rochester, NY) and exposed to X-ray at 30 kV for 10 s in a Fixitron radiographic inspection unit (model 43B55A). Exposed films were developed using an automatic film processor (Kodak RP X-OMAT). Radiographs of bones were evaluated for the presence of tumor foci.

**Histological Examinations.** Animals were sacrificed with CO₂. The lung, heart, liver, kidney, spleen, pancreas, forelimbs, and hind limbs were incised and fixed with 10% formalin. The bone tissues were decalcified in Cal-Ex II solution (Fisher Scientific) for 24 h. All tissues were embedded in paraffin. Histological sections were prepared by standard conventional processing and stained with H&E. Micrographs were taken with a Nikon microscope equipped with a digital camera (Cool Snap) and further processed using Adobe Photoshop software.

**RESULTS**

**Preparation of MDA-MB-231 Cells Infected with Retrovirus Carrying GFP-cortactin Variants.** To examine the role of cortactin in tumor progression, wild-type cortactin and a tyrosine phosphorylation-deficient cortactin mutant (19) were tagged at their NH₂ termini by GFP in retroviral vector MGIn as described previously (12), yielding fusion proteins GFP-cortactin and GFP-CortF₄₂₁F₄₆₆F₄₈₂, respectively. The viruses carrying GFP-cortactin and GFP-CortF₄₂₁F₄₆₆F₄₈₂ were used to infect MDA-MB-231 cells. The infected cells were further enriched up to 85% by FACs based on the expression of GFP. Expression of GFP-cortactin proteins was further quantified by immunoblot analysis. As shown in Fig. 1, both GFP-cortactin and GFP-CortF₄₂₁F₄₆₆F₄₈₂ were expressed at a level nearly 2-fold higher than endogenous cortactin. The epitope GFP did not seem to affect the specificity of tyrosine phosphorylation of cortactin because GFP-cortactin, but not GFP-CortF₄₂₁F₄₆₆F₄₈₂, was able to be phosphorylated in response to a mixture of hydrogen peroxide and sodium vanadate (Fig. 1). A similar response was also described with either endogenous cortactin or small epitopes such as Myc-tagged cortactin proteins (19).

**Overexpression of GFP-Cortactin Variants Did Not Affect the Growth of Breast Cancer Cells.** To study the effect of cortactin on cell proliferation, the growth of MDA-MB-231 cells infected with GFP-cortactin viruses in either normal culture medium, soft agarose, or the mammary fat pad of nude mice was evaluated. None of these GFP-cortactin viruses in either normal culture medium, soft agarose, cell proliferation, the growth of MDA-MB-231 cells infected with GFP-cortactin, and GFP-CortF₄₂₁F₄₆₆F₄₈₂ was expressed at a level nearly 2-fold higher than endogenous cortactin. The epitope GFP did not seem to affect the specificity of tyrosine phosphorylation of cortactin because GFP-cortactin, but not GFP-CortF₄₂₁F₄₆₆F₄₈₂, was able to be phosphorylated in response to a mixture of hydrogen peroxide and sodium vanadate (Fig. 1). A similar response was also described with either endogenous cortactin or small epitopes such as Myc-tagged cortactin proteins (19).

**Overexpression of GFP-Cortactin Variants Potentiates Bone Metastases.** MDA-MB-231 cells infected with GFP-cortactin variants were also evaluated for their metastatic ability by an experimental bone metastasis assay involving injection of the cells into the left cardiac ventricles of nude mice (20). Five weeks after injection, the animals bearing GFP-cortactin cells developed apparent cachexia (loss of muscle, fat, and body weight). These animals had an average body weight that was 25% less than that of control animals injected with cells expressing the viral vector only (Fig. 3A). In contrast, the average body weight of mice bearing GFP-CortF₄₂₁F₄₆₆F₄₈₂ was about 20% higher than that of the control animals.

The tumors that had metastasized into bone tissues were examined by X-ray radiography, which revealed tumor-induced bone lesions as radiolucent foci (Fig. 3C). As summarized in Fig. 3B, the mice bearing GFP-cortactin cells developed an average of 7 tumors/animal, whereas the mice bearing GFP only developed an average of <4.
tumors/animal. In contrast, the mice injected with GFP-Cort F421F466F482 cells showed much less potential for bone metastasis and developed only 1 tumor/animal. This result is consistent with our previous finding that the mutant acts in a dominant negative manner within cells (12). Tumors grown in other tissues were also examined by histological analysis. Most tumor metastases were found within bone tissues, a few in were found in the lung, and one was found in the adrenal gland (Table 1). No tumors were found in the heart, kidney, spleen, liver, and pancreas.

Overexpression of GFP-cortactin Variants Modulates Transendothelial Invasion of MDA-MB-231 Cells. We also attempted to examine whether the enhancement of tumor metastases manifested by overexpression of cortactin could be attributable to an increase in invasion through endothelial cells (21). Tumor cells were placed on the monolayer of human BMECs grown in the top chamber of Transwell, a modified Boyden chamber apparatus. After 20 h, the cells that had migrated through the membrane on the bottom chamber were examined by fluorescence microscopy. As shown in Fig. 4A, GFP-cortactin cells exhibited a motility 70% higher than that of the control cells, whereas the motility of GFP-Cort F421F466F482 cells was 45% lower than that of the control cells (Fig. 4A).

The apparent cortactin-induced increase in transendothelial invasion could be the result of an increase in the adhesion of tumor cells to endothelial cells. The adhesive abilities of tumor cells were evaluated by measuring the number of cells that were able to attach to the monolayer of human BMECs 4 h after plating. As shown in Fig. 4B, GFP-cortactin cells enhanced the adhesive affinity for endothelial cells by ~62% compared with cells expressing the viral vector alone, whereas GFP-Cort F421F466F482 cells adhered to human BMECs with an efficiency that was 40% lower than that of the control cells.

DISCUSSION

Metastasis is a multistage process and requires tumor cell invasion into lymphatic and blood circulation systems, adhesion to endothelium, transendothelial invasion and migration, and, finally, colonization in distant tissues (22). Whereas the detailed molecular mechanism for each process remains unclear, tumor invasion requires the formation of protrusions projected from the membrane surface of tumor cells (23). These pseudopods, which are also called invadopodia (24), may facilitate either the action of proteinases for the degradation of extracellular matrix around the tumors or the penetration of cells into matrix.
contact regions between endothelial cells, eventually enabling the entire cells to invade through the endothelial layer (23). Thus, a molecule that is implicated in the formation of cellular protrusions and cell adhesion to endothelial cells would likely be a good candidate for playing a role in metastasis. This study provides the first evidence that cortactin, a cell protrusion-associated protein, is able to enhance tumor metastasis in vivo. Overexpression of wild-type cortactin in MDA-MB-231 cells significantly enhanced the frequency of bone metastasis in nude mice (Fig. 3). This result was consistent with reports that overexpression of cortactin via gene amplification is often associated with poor prognosis of cancer patients (9, 25).

Cortactin could play a role in early stages of tumor progression because its phosphorylation level is known to be up-regulated by oncogenes and growth factors (26, 27). However, cells overexpressing either wild-type or tyrosine phosphorylation-deficient cortactin variants had a growth rate similar to that of control cells expressing the vector only as analyzed by growth curve, colony formation in soft agarose, and growth in the mammary fat pad (Fig. 2). This result agrees with our previous finding (6) that Src(−/−) cells, in which tyrosine phosphorylation of cortactin is impaired, exhibit a response similar to that of normal cells to fibroblast growth factor for cell growth. Thus, overexpression of cortactin via gene amplification may serve as a pathogenic mechanism in the late stages of tumor development rather than contribute directly to the primary tumor progression, which is known to involve many genes responsible for cell cycle regulation (28). However, the pathological function of cortactin may be implemented in concert with oncogenes that are involved in cell growth. In this regard, it is worth noting that cortactin is frequently coamplified in cancers with cyclin D1, an important regulator of the cell cycle (4).

Overexpression of cortactin can increase by 62–70% in transendothelial invasion as well as adhesion to endothelial cells (Fig. 4). Adhesion of tumor cells to endothelial cells is known to be an important step in tumor metastasis and may determine the rate of cell transendothelial invasion (29). Indeed, highly metastatic colorectal cancer cells also tend to have higher affinities for endothelial cells than do poorly metastatic cells (30). However, the mechanism by which overexpression of cortactin facilitates the interaction of tumor cells with endothelial cells is not clear. It may involve the activation of cell surface proteins implicated in cell adhesions via alteration of the actin cytoskeleton underneath the plasma membrane, which is required for the formation of cell-to-cell or cell-to-extracellular matrix interactions (31). The actin cytoskeleton is a dynamic structural entity and constantly undergoes assembly or disassembly (32). The assembled actin filaments can be further cross-linked to form either actin bundles or actin meshwork. Cortactin may alter the cytoskeleton by its ability to promote actin assembly via activation of Arp2/3, a protein complex that plays a vital role in the nucleation of actin assembly (13), and to cross-link actin filaments in a reversible manner dependent on its tyrosine phosphorylation (5). Cortactin also contains a COOH-terminal SH3 domain, which is known to bind to various membrane-associated proteins including ZO1, a junction-associated protein (33). Thus, cortactin may link a cell adhesion molecule either directly or indirectly via its SH3 domain.

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