E-Cadherin Expression in Human Breast Cancer Cells Suppresses the Development of Osteolytic Bone Metastases in an Experimental Metastasis Model¹

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ABSTRACT

The molecular mechanisms by which human cancer cells spread to bone are largely unexplored. The process likely involves cell adhesion molecules (CAMs) that are responsible for homophilic and heterophilic cell-cell interactions. One relevant CAM may be the calcium-dependent transmembrane glycoprotein E-cadherin. To investigate the involvement of E-cadherin in breast cancer metastasis to bone, we used an in vivo model in which osteolytic bone metastases preferentially occur after injections of cancer cells directly into the arterial circulation through the left ventricle of the hearts of nude mice. We have found that E-cadherin-negative human breast cancer cells MDA-MB-231 (MDA-231) develop radiographically detectable multiple osteolytic bone metastases and cachexia in this model. However, MDA-231 breast cancer cells that were transfected with E-cadherin cDNA showed a dramatically impaired capacity to form osteolytic metastases and induce cachexia. Histological and histomorphometrical analyses of bones of mice bearing mock-transfected MDA-231 revealed aggressive metastatic tumor, whereas metastatic tumor burden was significantly decreased in the bones of mice bearing E-cadherin-expressing MDA-231. Nude mice bearing E-cadherin-transfected MDA-231 breast cancer cells survived longer than mice bearing mock-transfected MDA-231 breast cancer cells. Anchorage-dependent and independent growth in culture and tumor enlargement in the mammary fat pad of nude mice were unchanged between mock-transfected and E-cadherin-expressing MDA-231, suggesting that these differences in metastatic behavior are not due to an impairment of cell growth and tumorigenicity. Our results show the suppressive effects of E-cadherin expression on bone metastasis by circulating breast cancer cells and suggest that the modulation of expression of this CAM may reduce the destructive effects of breast cancer cells on bone.

INTRODUCTION

E-cadherin (uvomorulin) is a M₄, 120,000 transmembrane glycoprotein involved in calcium-dependent cell-cell adhesion. Cell-cell adhesion mediated by E-cadherin is homophilic or heterophilic, causing homotypic or heterotypic cell aggregation, which is likely important in controlling embryogenesis, morphogenesis, and tissue-specific immunity (1, 2). Recently, evidence has accumulated that E-cadherin also plays a key role in cancer invasion and metastasis. Immunohistochemical examination of clinical tumor specimens of the head and neck (3), prostate (4), stomach (5), kidney (6), and other organs (7) has shown that E-cadherin expression is decreased in invasive and metastatic cancers and that decreased E-cadherin expression is associated with poor prognosis (6, 8). It has also been shown that poorly differentiated metastatic breast cancers express lower levels of E-cadherin than do well-differentiated breast cancers (9–11). These clinical studies suggest that E-cadherin expression is inversely correlated with the invasiveness and metastatic potential of cancer. However, they do not show whether E-cadherin expression affects the behavior of cancer cells per se during invasion and metastasis.

Treatment of noninvasive epithelial Madin-Darby canine kidney cells with monoclonal antibodies to E-cadherin in vitro rendered these cells more invasive (12). Expression of the E-cadherin gene in highly invasive cancer cells dramatically suppressed their invasiveness and, conversely, introduction of E-cadherin-specific antisense RNA rendered noninvasive epithelial cells invasive in vitro (13). E-cadherin was strongly expressed in breast cancer cells with reduced invasiveness, whereas relatively low levels of E-cadherin were detected in highly invasive human breast cancer cells (14). These experimental results show the inhibitory effects of E-cadherin on the local invasiveness of cancer cells and are consistent with the notion that E-cadherin is an “invasion suppressor gene” (13). However, they do not demonstrate whether E-cadherin expression also suppresses cancer metastasis to distant organs. Because multiple sequential steps are required for cancer cells to advance to achieve distant metastasis, it is likely that the expression of E-cadherin inhibits detachment and invasion of cancer cells at the primary sites before the step of intravasation. Conversely, however, it is also possible that, subsequent to intravasation, E-cadherin may rather facilitate the arrest of cancer cells at metastatic sites or coaggregation of cancer cells with platelets in capillaries, resulting in a promotion of metastasis. Determining whether this is the case seems to be important to our understanding of the role of E-cadherin expression in distant metastasis of cancer.

We have recently developed an experimental metastasis model in nude mice in which inoculation of cancer cells into arterial circulation through the left cardiac ventricle selectively results in osteolytic bone metastases (15), based on the method described by Arguello et al. (16). Using this model of bone metastasis, we examined the role of E-cadherin in bone metastasis of human breast cancer because breast cancer frequently colonizes bone (17, 18). The E-cadherin-negative human breast cancer cell line MDA-231 was transfected with mouse E-cadherin cDNA and studied for the capacity of these cells to develop osteolytic bone metastases. Our data provide in vivo experimental evidence that functional E-cadherin expression in MDA-231 breast cancer cells caused a marked decrease in osteolytic bone metastases even after these cells were directly introduced into the blood stream. The results suggest that E-cadherin might have suppressive effects not only on the local invasiveness of cancer cells at primary sites but also on the metastasis of circulating cancer cells to distant organs such as bone.

MATERIALS AND METHODS

MDA-231: A Human Breast Cancer Cell Line. The E-cadherin-negative human breast cancer cell line MDA-231 (Ref. 19; kindly provided by Dr. C. Kent Osborne, University of Texas Health Science Center, San Antonio, TX) was cultured in DMEM (Hazelton Biologies, Inc., Lenexa, KS) supplemented with 10% FCS (Hyclone Laboratories, Logan, UT) and 1% penicillin-streptomycin solution (Life Technologies, Inc., Grand Island, NY) in a humidified atmosphere of 5% CO₂ in air.

The abbreviations used are: MDA-231, MDA-MB-231 human breast cancer cell line; MMP, matrix metalloproteinase; TBS, Tris-buffered saline.

Received 4/22/96; accepted 7/29/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by NIH Grants PO1-CA40035, CA58183 (Specialized Program of Research Excellence), and CA63628.

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Transfection of Mouse E-Cadherin cDNA. MDA-231 (1 × 10^5) cells grown in DMEM supplemented with 10% FCS were cotransfected with 20 μg of full-length mouse E-cadherin cDNA (in pBATEM2 plasmid driven under the control of the chicken β-actin promoter; Ref. 20) and 1 μg of pSV2neo (21) for neomycin resistance by calcium-phosphate coprecipitation method (22). As a control, cells were also transfected with 2 μg of pSV2neo. The E-cadherin expression plasmid, pBATEM2, was a generous gift of Dr. M. Takeichi (Kyoto University, Kyoto, Japan). The neomycin expression vector, pSV2neo, was purchased from Invitrogen (San Diego, CA). After 24 h of incubation, cells were fed with fresh medium, grown for an additional 24 h, split and seeded into 100-mm dishes, and cultured in the presence of 470 μg/ml of antibiotic G418 (Life Technologies, Inc.). G418-resistant colonies were isolated and cloned by limiting dilution twice.

Immunofluorescent Staining of E-Cadherin. Cells were fixed with a fresh solution of 3.7% (w/v) formaldehyde in PBS at room temperature for 30 min, incubated with the primary antibodies (ECCD-2; Zymed Laboratories, Inc., San Francisco, CA) at a 1:200 dilution in PBS containing 1% rabbit serum at 37°C for 1.5 h in humidified atmosphere, washed three times with PBS, and incubated with 1:80-diluted affinity-purified rabbit anti-rat IgG conjugated with fluorescein 5-isothiocyanate (Sigma Chemical Co., St. Louis, MO). After washing with PBS, cells were mounted in PBS-glycerol solution (1:1) and examined under a fluorescence microscope (Nikon Inc. Instrument Division, Garden City, NY).

Immunoblotting. Cells were lysed in lysis buffer [20 mM Tris (pH 8.0), 2 mM CaCl_2, 150 mM NaCl, 1% NP40, 0.1% SDS, and protease inhibitors (20 mM leupeptin, 1 mM phenylmethylsulfonylfluoride, and 1% aprotinin)] at 4°C and centrifuged at 10,000 × g for 10 min at 4°C, and protein amounts were determined using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA). The lysates were boiled for 5 min, separated on 7.5% SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA) in transblotting buffer [20 mM Tris, 150 mM glycine, and 20% methanol (pH 8.0)]. The membranes were incubated overnight with blocking buffer [5% BSA in 50 mM Tris, 150 mM NaCl, and 10 mM CaCl_2] and then with monoclonal antibodies to mouse E-cadherin (Transduction Laboratories, Lexington, KY) diluted 1:5000 in the same buffer for 2 h, washed once with TBS + 0.05% Tween 20 (TBST), then twice with TBS, and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Capel, Durham, NC; diluted 1:1500 in TBS containing 5% nonfat dry milk) for 1 h. After the membranes were washed three times with TBST and then three times with TBS, the signal was visualized with an enhanced chemiluminescence detection system (DuPont NEN, Boston, MA).

Colonies Growth in Monolayer. Cells (1 × 10^5/well, 6-well plate; Falcon; Becton Dickinson Labware, Lincoln Park, NJ) were cultured in DMEM supplemented with 10% FCS. After the indicated time of culture, cells were trypsinized and counted on a hemocytometer under an inverted microscope.

Intracardiac Injections of MDA-231 Cells in Nude Mice. Intracardiac injection of MDA-231 cells was performed according to the technique described previously (24). Subconfluent MDA-231 cells were fed with fresh medium 24 h before intracardiac injections. Cells (1 × 10^5) were suspended in 0.1 ml of PBS and injected using a 27-gauge needle into the left heart.
Determination of the Number and Area of Bone Metastases. The number of osteolytic bone metastases was enumerated on radiographs as described (15, 24). Animals were anesthetized deeply, placed in prone and lateral positions against the films (22 × 27 cm; X-OMAT AR; Kodak, Rochester, NY), and exposed to an X-ray at 35 kV for 6 s using a Faxitron radiographic inspection unit (Model 43855A; Faxitron X-ray Corporation, Buffalo Grove, IL). Films were developed using a Kodak RP X-OMAT processor (Model M6). All of the radiographs of bones in nude mice were evaluated extensively and carefully by three individuals (including one radiologist) who had no knowledge of the experimental protocols. On radiographs, the number and area of osteolytic metastatic foci as small as 0.5 mm in diameter, which were recognized as demarcated radiolucent lesions in the bone, were quantitatively assessed using a computer-assisted Jandel Video Analysis (JAVA) image analysis system (Jandel Scientific, Corte Madera, CA).

Tumorigenicity of MDA-231 Cells in Nude Mice. Cells (2 × 10^6) were suspended in 0.2 ml of the mixture (1:1) of PBS and Matrigel (Collaborative Research, Bedford, MA) and inoculated into the right thoracic mammary fat pad of female nude mice using 23-gauge needles as described previously (26). Four weeks later, the tumors formed were excised and their weights were determined.

Histological, Histomorphometrical, and Stereological Examination of Metastatic Cancer Burden. The details of these methods were described previously (24). In brief, forelimbs and hind limbs from animals in each treatment group were fixed with 10% formalin in PBS (pH 7.2) and decalcified in 14% EDTA solution for 2–3 weeks. Paraffin sections were made using conventional methods. The area of metastatic cancer infiltrations was measured in the distal femoral and proximal tibial metaphyses of both limbs in longitudinal decalcified sections stained by H&E. A level showing the full width of the growth plate and shaft was selected for measurement. All metastatic cancer cells from the joint surface to a point 4 mm down the shaft were measured in both the bone and where it had expanded into the surrounding soft tissues. Metastases in the epiphyses were also included.

Statistical Analysis. All data were analyzed by Mann-Whitney test for nonparametric samples. The statistical difference of survival rate of the animals was analyzed by generalized Wilcoxon test. All data were presented as the mean ± SE.

RESULTS

Transfection of E-Cadherin cDNA into MDA-231 Cells. Subsequent to transfection, G418 selection, and cloning by limiting dilution, several subclones of MDA-231 breast cancer cells were obtained. MDA-231 subclones transfected with E-cadherin cDNA were designated E-cad-MDA-231, and MDA-231 subclones transfected with pSV2neo were designated neo-MDA-231 and used as controls in the following experiments. E-cad-MDA-231 cells expressed E-cadherin

ventricles of 4-week-old female BALB/c- nu/nu mice (Harlan Industries, Houston, TX) under anesthesia with pentobarbital (0.05 mg/g). Animals were kept in our animal facilities for 4–7 weeks as described (25). The weight of animals and excised tumors was determined by using a Lum-O-Gram™ (Ohaus Scale Corporation, Florham Park, NJ).
E-cadherin expression affects the growth of MDA-231 cells, the growth curve of E-cad-MDA-231 cells was compared to that of neo-MDA-231 cells in monolayer culture. There was no difference in the growth pattern between E-cad-MDA-231 and neo-MDA-231 cells (Fig. 2A). Furthermore, anchorage-independent growth of neo-MDA-231 and E-cad-MDA-231 cells assessed by colony formation in soft agar was not significantly different (Fig. 2B).

More importantly, the weight of E-cad-MDA-231 tumors formed in the mammary fat pad of nude mice 4 weeks after the inoculation was not different from that of neo-MDA-231 tumors (Fig. 2C). These in vitro and in vivo results convincingly demonstrate that transfection of E-cadherin cDNA did not impair the capacity of growth and tumorigenicity of MDA-231 cells.

Development of Cachexia and Osteolytic Bone Metastases. Nude mice injected with neo-MDA-231 cells into the left ventricle of the heart showed profound cachexia (loss of muscle, fat, and body weight; Fig. 3 and Fig. 4, top) and multiple osteolytic lesions in the upper and lower extremities (Fig. 4, bottom and Fig. 5) 3–4 weeks after inoculation, as we reported previously (15, 24). On the other hand, nude mice inoculated with E-cad-MDA-231 cells demonstrated a marked impairment of cachexia (Fig. 3) and a significant decrease in osteolytic bone metastases (Fig. 4, bottom and Fig. 5).

Histological View of Bones with Metastatic MDA-231 Cells. Neo-MDA-231 cells almost completely destroyed the marrow architecture, including the primary and secondary spongiosa of tibial bone, and occupied the entire marrow cavity with frequent extension to the surrounding soft tissue (Fig. 6). In contrast, E-cad-MDA-231 colonization was relatively limited in the marrow cavity, and the majority of trabecular bone and primary and secondary spongiosa remained intact (Fig. 6). In both groups, enhanced osteoclastic bone resorption along the endosteal surface of bone was observed adjacent to metastatic MDA-231 cells (Fig. 6). The osteoclast number and bone resorption surface area determined by histomorphometry were not different in bones of E-cad-MDA-231- and neo-MDA-231-bearing mice (data not shown).

Histomorphometrical Analysis of Metastatic Cancer Burden in Bone. Consistent with the histological view, histomorphometrical analysis of bones with neo-MDA-231 cells showed a larger metastatic cancer burden than bones with E-cad-MDA-231 cells (Fig. 7).

Metastasis in Non-Bone Organs. As a characteristic feature of this experimental metastasis model and by yet-unexplained mecha-

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Fig. 4. Representative pictures (top) and radiographs (bottom) of nude mice bearing neo (left) and E-cad (right; subclone 4 of Fig. 1, top)-MDA-231 cancer. Arrows, osteolytic bone metastases. Pictures and radiographs were taken 4 weeks after cell inoculation. Note the differences in cachexia (loss of fat, muscle, and body weight) and the number of osteolytic bone metastases between both groups.

Fig. 5. The number and area of osteolytic bone metastases in nude mice bearing neo (○)- and E-cad (■)-MDA-231 cancer. The number and area of osteolytic bone metastases were scored 4 weeks after cell inoculation on radiographs using quantitative image analysis. Each group had five mice. Values shown are mean ± SE (n = 10). *, significantly different from the neo-MDA-231 group (P < 0.005).
Fig. 6. Histological view of the proximal tibiae of nude mice bearing no cancer (A), neo-MDA-231 (B), and E-cad-MDA-231 (C; subclone 4 of Fig. 1, top). Almost all the primary and secondary spongiosa were replaced by metastatic breast cancer (T) in mice inoculated with neo-MDA-231 (B) as compared to the bone of non-tumor-bearing normal mice (A). In contrast, colonization of E-cad-MDA-231 (T) in the tibiae was localized, and the majority of the marrow cavity remained intact (C), with an appearance similar to that of non-tumor-bearing normal mice. H&E staining. ×40. Osteoclastic bone resorption along the endosteal surface of bone of nude mice bearing neo-MDA-231 (D) and E-cad-MDA-231 (E) at higher magnification. Osteoclasts (arrows) are present between metastatic MDA-231 cells (T) and bone. H&E staining. ×200.

nisms, very few metastases in other organs such as lungs, kidney, brain (data not shown), and liver were detected in both groups (Table 1). Therefore, it was not possible to statistically analyze the effects of E-cadherin expression on MDA-231 metastasis to non-bone organs.

Survival of Animals. Nude mice inoculated with E-cad-MDA-231 cells survived significantly longer than animals injected with non-transfected MDA-231 cells or neo-MDA-231 cells (Fig. 8). However, it should be noted that all animals bearing E-cad-MDA-231 cells also died of cancer with osteolytic bone metastases (with analogous radiographical and histological views to those of neo-MDA-231-bearing animals) 8 weeks after the inoculation. The results indicate that E-cad-MDA-231 cells did not lose the capacity to develop bone metastases, but that the capacity is impaired, presumably due to E-cadherin expression.

Production of Bone-destroying Activity. To determine whether E-cad-MDA-231 cells produce greater levels of osteolytic activities than do neo-MDA-231 cells, serum-free culture supernatants were
E-cadherin in E-cadherin-negative MDA-231 human breast cancer cells suppresses metastasis to bone even after the cells have entered the systemic circulation, diminishes loss of body weight, and prolongs the life span of MDA-231 breast cancer-bearing animals. The results provide experimental evidence that E-cadherin has a suppressive action on distant metastasis of breast cancer cells to bone in vivo.

The mechanisms by which E-cadherin expression decreases osteolytic bone metastases in this animal model remain to be elucidated. In this study, several subclones of MDA-231 cells that were transfected with E-cadherin cDNA exhibited decreased formation of osteolytic bone metastases, indicating that the results obtained here reflect the phenotype of E-cadherin-expressing MDA-231 cells and are not due to the clonal difference. Our data also demonstrate that E-cadherin expression had no effects on the growth and tumorigenicity of MDA-231 cells. Fri xen and Nagamine (31) have reported that E-cadherin expression is associated with decreased secretion of proteolytic enzymes. In our study, however, there was no change in MMP secretion and bone-destructing activity in the absence or presence of E-cadherin expression. Subsequent to entry into the systemic circulation, there are still multiple diverse and complex steps including directional migration, escape from host immune cell surveillance, and extravasation through which cancer cells must progress before they arrest in bone. One mechanism may involve the alteration of cell-cell adhesion between MDA-231 cells. E-cad-MDA-231 cells in culture demonstrated changes in morphology and increased cell-cell contact. Clumping or clustering of MDA-231 cells may affect dispersion and migration capacity in the circulation and reduce cancer cell passage through bone capillary beds. Recent studies have described that much stronger shear forces are required to dissociate the aggregates of E-cadherin-positive breast cancer cells than E-cadherin-negative breast cancer cells (32) and that E-cadherin expression is inversely correlated with cell motility (33). E-cadherin has been shown to mediate not only homophilic but also heterophilic and heterotypic cell-cell contact (34) and cell-substratum adhesion (35). Therefore, it is possible that the interactions of MDA-231 cells with host cells and microenvironments are modulated by E-cadherin expression, leading to decreased bone metastases. To further study the role of E-cadherin in MDA-231 metastasis to bone, we are currently examining chemotactic migration, invasiveness, and adherence to various types of extracellular matrix in neo- and E-cad-MDA-231 cells, using Boyden chamber assay, Matrigel, and extracellular matrix-coated culture plates, respectively.

There is a characteristic step in bone metastasis that is not relevant in metastasis to non-bone organs. Because the majority of bone consists of calcified matrix and cancer cells do not apparently possess an intrinsic capacity to destroy bone, the cooperative assistance of osteoclasts, the only cells capable of resorbing bone, is required for cancer cells to grow progressively in bone (17, 18). Thus, as a mechanism specific for bone metastasis, a functional association between metastatic cancer cells and osteoclasts that are modulated by either soluble factors or direct cell-cell contact or both in the bone microenvironment seems to be necessary for the development of osteolytic metastases. Along this line, it is noteworthy that our recent data demonstrate that osteoclasts express E-cadherin (27). However,

Table 1 Dissemination of MDA-231 in the organs of nude mice

<table>
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<th>Organ</th>
<th>Neo-MDA-231 (n = 15)</th>
<th>E-cad-MDA-231 (n = 15)</th>
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<td>Adrenal glands</td>
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MDA-231 cells (1 × 10^6) were inoculated into the left cardiac ventricle. Four weeks after the inoculation, each organ was examined macroscopically and microscopically. (+), metastasis-positive; (−), metastasis-negative; (±), occasionally metastasis-positive.
our results demonstrated that E-cadherin expression did not alter the production of osteolytic activity and MMP secretion in MDA-231 cells in culture. Thus, it seems unlikely that E-cadherin expression modulates the secretion of soluble factors by metastatic MDA-231 breast cancer cells that stimulate osteoclastic bone destruction.

Enhanced cell-cell aggregation in E-cad-MDA-231 cells also indicates that the transfected E-cadherin cDNA is functional, and that downstream signaling molecules, including catenins (36), are intact in MDA-231 cells. In fact, a recent study has demonstrated moderate expression of α- and β-catenin in MDA-231 cells (37). Because cadherin-mediated cell-cell adhesion requires an association of catenin with catenins (36) and evidence that catenins also play a critical role in cancer invasiveness and metastasis is accumulating (38—41), the involvement of catenins in MDA-231 metastasis to bone needs to be explored.

In conclusion, we have found in the present study that the expression of E-cadherin inhibits the metastasis of circulating human breast cancer cells to bone in vivo. Inhibition of α,β3 integrin by peptide antagonists or monoclonal antibodies has been demonstrated to promote primary tumor regression by inhibiting angiogenesis (42). In addition to integrins, the modulation of expression of another cell adhesion molecule, E-cadherin, by administering agents that up-regulate E-cadherin expression may be a potential therapeutic approach to inhibit the spread and destructive effects of breast cancer in bone. Our results support the feasibility of such a strategy.

ACKNOWLEDGMENTS

We thank Drs. M. Takeichi and R. Kessler for providing us with plasmid pBATEM2 and DECMA-1 antibodies, respectively, and Thelma Barrios for secretarial assistance.

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