Abstract. Aim: The aim of the present study was to evaluate E-cadherin, whose expression remains poorly understood in the intercellular adhesion of metastatic breast cancer cells in bone, the most prevalent site for metastatic growth. Materials and Methods: An immunohistochemical staining method was used for the localization of E-cadherin protein in tissue biopsy specimens of normal breast (n=9) and well- (n=8), moderately (n=8) or poorly (n=14) differentiated invasive primary breast cancer and metastatic breast cancer in bone (n=17). The expression patterns of E-cadherin were classified as homogeneous (most cells exhibiting positivity), heterogeneous (a few scattered patches of cells with positivity) or negative (cells with undetectable positivity). Results: Normal breast epithelial cells showed homogeneous overexpression of E-cadherin in all cases. A progressive and statistically significant reduction of E-cadherin expression was detected in the histologically well- to moderately to poorly differentiated breast cancer cells (p<0.001). The clumps of invasive primary breast cancer cells in CD-31-positive blood vessels exhibited E-cadherin expression. Moreover, as compared to the poorly differentiated breast cancer cells, a significantly increased frequency of the metastatic breast cancer cells in bone exhibited homogeneous expression of E-cadherin in 15 out of 17 and heterogeneous expression in the remaining 2 cases (McNemar Exact p<0.001). This is the first demonstration of membranous overexpression of E-cadherin on metastatic breast cancer cells in bone; the high frequency of its expression may have a role in the intercellular adhesion of metastatic cells in bone.

Mortality of patients with invasive breast cancer occurs mostly due to spread and growth of breast cancer cells to distant organs such as bone, brain, lung or liver. The growth of the metastatic cancer cells in these organs disrupts their normal and vital function, leading to the life-threatening stages of the disease (1). One of the important aspects for the development of treatment strategies for metastatic breast cancer is to elucidate the mechanisms of the intercellular adhesion of growing cancer cells in bone, which is the most prevalent site for the growth of metastatic breast cancer cells (1, 2). However, inadequate information is available about the molecules that are involved in the intercellular adhesion of metastatic breast cancer cells at distant organs.

One of the critical intercellular adhesion proteins, E-cadherin, maintains the cellular polarity and morphological structure of normal epithelial cells including that of the breast. E-cadherin is a transmembrane glycoprotein and an epithelial-specific member of the cadherin family of intercellular adhesion molecules. It mediates an epithelial cell–cell interaction through calcium-dependent homophilic interaction of its extracellular domain (3, 4). The cytoplasmic domain of E-cadherin forms complexes with - and -catenins and p120, and the complexes thus formed interact with the actin cytoskeleton to maintain differentiated architecture and polarity of normal epithelial cells in tissue (4-7). Loss of the functional interaction between E-cadherin and -catenin not only disrupts intercellular adhesion of invasive cancer cells, but also alters the signaling mechanism that regulates cellular growth and differentiation (8).

Indeed, a reduction or loss of membranous expression of E-cadherin expression on invasive cancer cells at the primary sites has been widely reported to be associated with high

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Key Words: Breast cancer, bone metastasis, E-cadherin, methylation, immunohistochemical staining.
Membranous expression of E-cadherin protein in the normal breast, primary breast carcinoma and breast cancer metastasis to the bone by an IHC staining method.

<table>
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<tr>
<th>Histology</th>
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<th>Heterogeneous</th>
<th>Negative</th>
<th>p-value</th>
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<td>0</td>
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<tr>
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<td>0</td>
<td>10</td>
<td>4</td>
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<tr>
<td>Bone metastasis</td>
<td>17</td>
<td>15</td>
<td>2</td>
<td>0</td>
<td>p&lt;0.001c</td>
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</table>

*aMembranous expression of E-cadherin protein was classified as homogeneously positive, heterogeneously positive or negative as defined in the Materials and Methods section. bFisher’s exact test; cExact McNemar test.

Histological grade, advanced clinical stage and poor prognosis of patients with adenocarcinomas that include breast (9-15), gastric (16-18), hepatocellular (19) and prostatic carcinomas (20-24), and squamous cell carcinoma of the head and neck (25, 26). Furthermore, a reduction or loss of membranous expression of E-cadherin protein on prostate cancer cells at the primary site has also been reported to be associated with the presence of metastases in lymph nodes (22, 24, 27, 28), and is hence suggested to be an invasion- or metastatic-suppressor gene product (29, 30). The results of these studies suggest that E-cadherin expression is important for the maintenance of cellular differentiation and polarity of normal epithelial cells in tissue, and that its down-regulation on the invasive cancer cells allows their detachment and migration from the primary sites as a possible early event in the process of metastases (27, 28). However, it seems improbable that the E-cadherin-negative metastatic breast cancer cells would adhere to one another to form large clusters of the growing cells at the metastatic sites.

Here we report the results of a study of immunohistochemical localization of E-cadherin protein in human normal breast cells, the primary invasive and the metastatic breast cancer cells in bone in tissue biopsy specimens.

Materials and Methods

Reagents. Mouse monoclonal anti-E-cadherin antibody and rabbit monoclonal anti-CD31 antibody were obtained from Zymed Laboratories Inc. (South San Francisco, CA, USA) and LabVision (Fremont, CA, USA), respectively. Avidin-biotin-peroxidase complexes (ABC) and biotinylated horse antimouse immunoglobulins, normal horse serum and normal goat serum were purchased from Vector Laboratories, Inc., Burlingame, CA, USA. Goat anti-mouse alkaline phosphatase (ALP)-labeled polymer and goat anti-rabbit horseradish peroxidase (HRP)-labeled polymer were obtained from Biocare Medical, Walnut Creek, CA, USA. The blocking peptides, specific for each of the two antibodies, were obtained from Biocare Medical, Walnut Creek, CA, USA. The blocking peptides, specific for each of the two antibodies, were obtained from Biocare Medical, Walnut Creek, CA, USA. All antibodies were diluted and used according to the vendors’ instructions. All other reagents used were of the highest purity available from Sigma Chemical Co., St. Louis, MO, USA.

Patients. The archival tissue biopsy specimens from 9 normal breast with no evidence of breast disease, from 30 well-documented patients with histologically well-, moderately or poorly differentiated primary breast carcinoma, and 17 patients with confirmed metastasis to bone were obtained (Table I). All the tissue specimens were obtained from the Department of Pathology, Chiangmai University, Chiangmai, Thailand, in accordance with the ethical policy and procedure of the University.

Single-antibody immunohistochemical staining. The tissue blocks were sectioned at 4 µm and representative sections were stained with hematoxylin and eosin to confirm the diagnosis prior to immunostaining as previously described (31). Endogenous peroxidase activity was quenched with H2O2 in methanol for 20 min. Following the incubation of slides with normal horse serum for 20 min to block nonspecific binding of the subsequent antibodies, sections were incubated overnight with 150 µl of mouse monoclonal anti-E-cadherin antibody (2.5 µg/ml). The biotinylated horse antimouse secondary antibody followed by avidin-biotin-peroxidase conjugate were applied to the sections. Diaminobenzidine (DAB) was used as the chromogen and hematoxylin as the counterstain. For each experiment, a control that consisted of the pre-absorbed primary monoclonal antibody with the appropriate blocking peptide (10 µg protein/ml of working dilution of the antibody) was included to determine specificity of the antibody reaction.

Immunohistochemical co-localization. The double-antibody immunohistochemical (IHC) staining system allows the simultaneous detection of two distinct antigens within the same or a different population of cells in contrasting colors: red using the alkaline phosphatase (ALP)-chromogen Fast Red and brown with the horseradish peroxidase (HPR)-chromogen DAB. Both colors allow for visualization against a hematoxylin counterstain, resulting in blue nuclear staining. Consequently, the method allows the most effective and objective means of distinguishing cells that express both proteins from those that express only one of the two proteins in contrasting colors.

Table I.
Step 1. The tissue blocks were sectioned at 4 μm and representative sections stained with H&E to confirm the diagnosis prior to immunostaining as described previously (31). Endogenous peroxidase activity was quenched with H2O2 in methanol for 20 min. Following the incubation of slides with normal goat serum for 20 min to block nonspecific binding of the subsequent antibodies, sections were incubated overnight with 150 μl (2.5 μg/ml) of the first primary mouse monoclonal anti-E-cadherin antibody, followed by incubation with the goat anti-mouse alkaline phosphatase (ALP)-labeled polymer. Following the incubation of the labeled polymer, the reaction was completed with incubation of the Fast Red substrate-chromogen which results in a red-colored precipitate at the target sites (antigen) of the first primary antibody.

Step 2. Upon completion of the first reaction, tissue sections were incubated with blocking agent that removes any potential cross-reactivity between the reaction along with blocking any endogenous alkaline phosphatase that may be present. Following the incubation with the blocking agent, the tissue sections were incubated with the second primary rabbit monoclonal anti-CD31 antibody at an appropriate concentration (2.0 μg/ml), followed by incubation with the goat anti-rabbit horseradish peroxidase (HRP)-labeled polymer. The reaction was completed with incubation with the DAB solution containing H2O2 which results in a brown-colored precipitate at the target sites (antigen) of the second primary antibody. Finally, the tissue sections were counterstained with hematoxylin, resulting in blue nuclear staining. For each experiment, tissue sections containing breast cancer cells or endothelial cells with known positive expression of E-cadherin or CD31 served as the positive controls. The application of primary antibody, preabsorbed with the appropriate and specific blocking peptide (10 μg/ml of working dilution of the antibody) was used as the control for the test of specificity.

Figure 1. Immunohistochemical staining of normal breast, primary breast carcinoma and metastatic breast cancer cells. Formalin-fixed and paraffin-embedded tissue sections were subjected to a single-antibody immunostaining with monoclonal anti-E-cadherin antibody or the double-antibody immunostaining with anti-E-cadherin and anti-CD31 antibody. Normal breast cells show expression of membranous E-cadherin protein (brown staining; a, arrow). In a representative case that consisted of histologically poorly differentiated primary breast carcinoma (b) and metastasis to the bone (d), the former exhibited mostly E-cadherin-negative cells (absence of brown staining; b, arrows) while the latter were E-cadherin-positive (brown staining; d, arrow). Small clusters of invasive breast cancer cells showed membranous E-cadherin expression (red staining; c, arrow) in CD31-positive blood vessels (brown staining; c, arrow) at the primary site in the breast. Cells in connective tissue of breast or bone were completely non-reactive with the antibody. The sections were counterstained with Harris’s hematoxylin (blue-color nuclear staining). Original magnification x310 (a-d).
Evaluation of immunohistochemical staining. The immunostained tissue sections were reviewed independently by two of us (S.A.I. and W.Y.N.) who were blind to the sequence of tissue specimens. The data of immunostaining analysis were stratified into three categories based upon the frequency of membranous expression patterns of E-cadherin, namely, homogeneous with most cells showing positivity, a pattern similar to that of normal breast cells (as shown in Figure 1a), heterogeneous with a few scattered patches of cells with positivity (as shown in Figure 1b) or negative (cells with undetectable positivity) as described elsewhere (26).

Statistical analysis. The membranous expression of E-cadherin protein was grouped as homogeneously positive, heterogeneously positive or negative as described above. Contingency tables and the Fisher’s exact test (32) were used to summarize the association between morphological differentiation and the E-cadherin protein expression in each of the tumor types. The McNemar test (33) was used to test the correlation between membranous expression of E-cadherin protein and presence of metastatic cancer cells in bone.

Results

Normal breast. The normal breast epithelial cells lining the ducts exhibited homogeneous expression of E-cadherin protein in all examined cases of the normal breast tissue specimens (Figure 1a, Table I). The normal breast cells were significantly associated with homogeneous membranous expression of E-cadherin (Fisher’s exact p<0.001). The specific antibody preabsorbed with the blocking peptide (E-cadherin) was non-reactive to the normal breast epithelial cells, attesting to the specificity of the reactivity of the antibody (result not shown). Under these conditions fibroblast, lymphocytes and other cells of connective tissue were consistently negative (Figure 1a).

Primary breast cancer. The pattern and frequency of E-cadherin protein expression on histologically well-differentiated breast carcinoma cells were similar to that of the normal breast epithelial cells (Table I). Homogeneous expression of E-cadherin was detected in 6 out of 8 of the histologically moderately differentiated breast carcinomas and heterogeneous expression in the remaining 2 cases (Table I). In contrast, poorly differentiated breast cancer cells did not exhibit homogeneous expression of E-cadherin in any of the 14 cases (Table I): these cells showed heterogeneous expression in 10 out of 14 and none in the remaining 4 cases (Figure 1b, Table I). A statistically significant inverse association was observed between membranous expression of E-cadherin protein and poor differentiation of primary breast carcinoma cells (Fisher’s exact p<0.001). The result is consistent with the previously reported observation by other investigators (9, 23). The cytoplasmic expression of E-cadherin protein was not observed in the cancerous or normal breast cells in this cohort of patients.

By the immunohistochemical co-localization method, mouse monoclonal anti-E-cadherin antibody exhibited membranous reactivity (red-color staining) with the clumps of invasive breast cancer cells in blood vessels, while rabbit monoclonal anti-CD31 antibody reacted with the membrane of endothelial cells (brown-color staining) lining the blood vessels (Figure 1c). The application of the pre-absorbed anti-cadherin antibody or anti-CD31 antibody with the specific blocking peptide led to the abolishment of the immunostaining, demonstrating the specificity of the antibody reactivity (result not shown).

Metastatic breast cancer cells in bone. A markedly higher frequency of metastatic breast cancer cells in bone showed homogeneous membranous expression of E-cadherin in 15 out of 17 cases (Figure 1d, Table I) and heterogeneous in the remaining 2 cases (Table I). A statistically significant association was obtained between membranous expression of E-cadherin protein and metastatic breast cancer cells in bone (Exact McNemar p<0.001).

Discussion

The expression of an important intercellular adhesion protein, E-cadherin, in normal human breast epithelial cells and its reduced or loss of expression in breast cancer cells at the primary site in breast tissue is well-documented (9, 15). However, the role of E-cadherin in metastatic breast cancer cells in distant organs remains poorly understood. Therefore, the goal of the study was to ascertain the expression of E-cadherin protein in breast cancer cells through their progression from the primary site in breast tissue to the distant metastatic site in bone tissue, which is the most prevalent site for the growth of breast metastatic cancer cells. The specimens of the normal breast tissue were used as the positive control for the membranous expression of E-cadherin protein, as this pattern of expression is known to reflect its intercellular adhesion function (8, 34).

A significantly high proportion of normal breast epithelial cells showed membranous expression of E-cadherin protein, confirming the previously reported results by other investigators (9, 11). In breast cancer, a progressive and statistically significant reduction of E-cadherin-positive cells was observed in the morphologically well- to moderately to poorly differentiated breast cancer cells. The pattern and frequency of E-cadherin protein expression in the normal breast cells and the primary breast cancer cells at various stages of morphological differentiation were consistent with the previously reported findings (9). However, it must be noted that even the small clumps of the cancer cells in CD31-positive blood vessels were positive for membranous expression of E-cadherin protein. This observation is not in
line with the prevailing notion that E-cadherin protein expression in cancer cells functions as a gatekeeper to prevent metastasis. In contrast to the moderately or poorly differentiated primary breast cancer cells, a significantly high proportion of metastatic breast cancer cells in bone exhibited membranous expression of E-cadherin protein. The frequency and pattern of E-cadherin expression on the metastatic cells were remarkably similar to those of the well-differentiated primary breast cancer cells and the normal breast cells. This is, indeed, a new observation that suggests the possibility of the functional expression of E-cadherin in the intercellular adhesion and formation of metastatic lesions in bone.

This new observation suggests two possible scenarios for the maintenance of E-cadherin protein expression in metastatic breast cancer cells in bone. In one possible scenario, E-cadherin protein expression is down-regulated in the primary cancer cells, probably due to an epigenetic mechanism, such as hypermethylation of its promoter region (12). The down-regulation of E-cadherin protein expression in the cancer cells would likely allow their detachment and migration from the primary site in the breast through the lymphovascular system to distant sites such as bone. In this scenario, E-cadherin is probably re-expressed through reversal of epigenetic silencing of the gene in the metastatic cells through their interactions with the host cell factors in bone. In another plausible scenario, E-cadherin protein expression is retained by a subpopulation of the primary breast cancer cells in small clusters which may enter the lymphovascular system, as has been observed in this study, to facilitate their metastatic migration to distant sites, culminating in their clonal growth in bone. Indeed, a complete absence of E-cadherin-positive cells was observed in only four cases of primary breast cancer, indirectly supporting the latter hypothesis.

The novel and paradoxical result of this study suggests that E-cadherin is unlikely to function as an invasion- or metastasis-suppressor gene product. A further study to elucidate the mechanism of the expression of E-cadherin protein on the metastatic breast cancer cells in bone may allow the development of strategies of therapeutic targeting to prevent the E-cadherin-mediated intercellular adhesion of growing metastatic cells.

References


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