Abstract. Background: Osteoclast activation plays an essential role in the development of bone metastasis (BM). Integrin αvβ3 mediates the attachment of osteoclasts to bone matrix, and it is overexpressed in bone-residing breast cancer cells. We studied BM from breast cancer in relation to its Integrin αvβ3 expression. Materials and Methods: Integrin αvβ3 expression in 4 human breast cancer cell lines (MDA-MB-231, MDA-MB-435, MKL-4 and T-47D) was determined by immunohistochemistry, flow cytometry and reverse transcriptase-polymerase chain reaction (RT-PCR). Results: Integrin αvβ3 was expressed in MDA-MB-231 and MDA-MB-435, but not in MKL-4 or T-47D, using both immunohistochemistry and flow cytometry. By RT-PCR, positive bands for both αv and β3 subunits were identified in MDA-MB-231 and 435, while only the αv subunit mRNA was present in MKL-4 and T-47D. BM was common in vivo following inoculation with MDA-MB-231 or 435, but not with MKL-4. Conclusion: Integrin αvβ3 expression appears to play a key role in the development of BM from breast cancer.

Bone is the most common metastatic site in breast cancer. Among primary breast cancer patients, 30% develop bone metastasis (BM) as the first event and 70% have BM at autopsy. However, the process of establishment of BM in bone and the bone marrow is not well understood. Integrins are alpha, beta heterodimeric transmembrane proteins. Integrins mediate adhesion to the extracellular matrix and cell-to-cell adhesive interactions (1). We know that Integrin αvβ3 mediates various biological events, including the adhesion of osteoclasts to the bone matrix and angiogenesis, among others. For osteoclastic activation in bone matrix to occur, osteoclasts must adhere to bone matrix through Integrin αvβ3. Helen et al. (2) showed that Integrin αvβ3 is overexpressed by bone-residing breast cancer cells and suggested either that subclonal selection of Integrin αvβ3-expression tumor cell populations or up-regulation of Integrin αvβ3 in the bone microenvironment must take place for BM to occur. Breast cancer cells adhere to the interstitial space of the bone matrix, but they lack the ability to resorb bone matrix by themselves. So breast cancer cells must be assisted by osteoclasts that can resorb bone matrix (3). If, by expressing Integrin αvβ3, cancer cells can adhere to bone matrix, they are likely to interact with osteoblasts and osteoclasts. In this study, we sought to determine whether Integrin αvβ3 is an adhesion molecule related to the establishment of BM from human breast cancer.

Materials and Methods

Animals. Eight-week-old nude rats were purchased and housed at the Laboratory Animal Center of Keio University, Japan, in a pathogen-free environment for the duration of the experiment. The Institutional Review Board of the animal facility approved the study protocol.

Cell lines. Four different breast cancer cell lines were studied: MDA-MB-231 (ATCC HTB 26), a human breast adenocarcinoma isolated from a pleural effusion; MKL-4, a cotransfectant of the MCF-7 cell line with fgf-4 and lacZ, which micrometastasises to several organs (gift of Dr. Kurebayashi, Kawasaki Medical School, Japan); T-47D (ATCC HTB 133), a human breast ductal carcinoma isolated from a pleural effusion; and MDA-MB-435 (ATCC HTB 129), a human breast ductal carcinoma isolated from a pleural effusion.

Cell culture. Cells were grown in Eagle's minimal essential medium (E-MEM) or Dulbecco's modified Eagle medium (D-MEM) in a gas mixture of 5% CO2 and 95% air at 37°C. Passage and medium exchange were performed once a week.

Immunohistochemistry. Cells on the slide were frozen and stained with anti-Integrin αvβ3 monoclonal antibody LM609 (1:100) and anti-Integrin αvβ1 monoclonal antibody BHA2.1 (1:100). The cells
were fixed in acetone for 30 sec, air-dried for 2 min, washed by Tris-buffered saline with calcium (TBS-Ca) for 2 min, blocked with 3% H₂O₂ for 10 min, washed twice with TBS-Ca for 5 min, and then incubated with LM609 or BHA2.1 overnight at 4°C. The next day, the slides were washed three times with TBS for 5 min, placed in ENVISION+ (Dako, Glostrup, Denmark) that had polymer-immuno complex at room temperature for 30 min, again washed three times with TBS for 5 min, and stained by diaminobenzidine (DAB) for 30 sec.

Flow cytometry. Cells were stained with 5 μg/mL mouse mAb LM609 specific to Integrin αvβ3 or mouse mAb BHA2.1 specific to Integrin α2β1 and incubated with anti-mouse-IgG FITC conjugate F(ab')2 Fragment (Sigma, St Louis, MO, USA). Viable cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Population gates were set using cells incubated with normal mouse IgG (4).

Reverse transcriptase–polymerase chain reaction (RT-PCR). RNA was isolated from human breast cancer cells. One microliter of cDNA was used per 25 μL of PCR reaction mixture. The reaction mixture contained TaKaRa Z-Taq, 10 X Z-taq buffer, dNTP mixture, MgCl₂ and PCR enhance (TaKaRa, Shiga-ken, Japan). We used the sequence reported in Baljit et al. (5) for αv and in Illera et al. (6) for β3. The 5’ primer for αv was 5’-GACTGTGTGGAAGACAATGTCTGTAAACCC, and the 3’ primer was 5’-CCAGCTAAGAGTTGAGTTCCAGCC; the size was 305 bp. These primers were extended from human αv cDNA (GenBank accession no. M14648). For β3, the 5’ primer was 5’-CTGGTGTTTACCACTGATGCCAAG, and the 3’ primer was 5’-TGTTGAGGCAGGTGGCATTGAAGG and the size was 393 bp. These primers were extended from human β3 cDNA (GenBank accession no. L28832). PCR amplification for αv was performed using a three-step process: i) initial extension at 94°C, for 1 min; ii) annealing at 65°C, for 1 min; and iii) final extension at 72°C, for 2
min, and for β3: i) initial extension at 95°C, for 10 min; ii) annealing at 60°C, for 2 min; iii) annealing at 72°C, for 3 min; and iv) final extension at 72°C, for 10 min. The amplified products were separated on 1% agarose gels using a 1-kilobase DNA ladder for size comparison.

Surgical procedure and evaluation of BM. The procedure was performed as described in detail elsewhere, with minor modifications (7). Briefly, after anesthetizing 8-week-old nude rats by intraperitoneal injection of ketaral 0.7 mL, a small incision was made in the left cervical skin under a dissecting microscope. A polyethylene catheter (PE-10, Becton Dickinson) was inserted into the thoracic aorta from the left common carotid artery. Cells (10^6 or 5x10^6 suspended in 0.2 mL of Hank's balanced salt solution) were injected slowly into the thoracic aorta. Then the catheter was removed, and the left common carotid artery was ligated.

Whole body radiographs were taken 4 and 6 weeks after injection to identify BM. X-ray confirmed the detection of the lesions (Figure 1).

Results

Immunohistochemical study identified the overexpression of Integrin αβ3 in two human breast cancer cell lines, MDA-MB-231 and MDA-MB-435, but not in MKL-4 or T47D (Figure 2). On flow cytometry, Integrin αβ3 was detected in 82% and 92% of MDA-MB-231 and MDA-MB-435 cell lines, respectively, and in 26% and 3% of MKL-4 and T-47D cells. αv and β3 subunit mRNA was isolated from each human breast cancer cell line by RT-PCR study. Positive bands for both the αv and β3 subunits were noted in MDA-MB-231 and 435, while only the αv subunit was detected in MKL-4 and T-47D (Figure 3).

These three studies were consistent in showing that Integrin αβ3 is expressed in MDA-MB-231 and MDA-MB-435, but not in MKL-4 or T-47D. Further, Integrin αβ3 was overexpressed in all human breast cancer cell lines on both immunohistochemical study and flow cytometry study (Table I).

In vivo, the frequency of developing BM using MDA-MB-231 cells was high [9/11 (81.8%)]. We were able to develop BM [8/10 (80.0%)] in rats using MDA-MB-435 cells, as well. Attempts to develop BM using MKL-4 failed [1/9 (11.0%)] (Table II).

Discussion

This study investigated the relationship between bone metastasis from breast cancer and Integrin αβ3. We previously reported that tartrate-resistant acid phosphatase (TRACP) is a useful marker for metastatic bone disease and of the response to treatment in breast cancer, and that both urinary pyridinoline and urinary deoxypyridinoline correlated with the volume of bone metastasis in a rat model (8). Those results showed that tumor cells initially produce micrometastatic foci in the bone marrow, followed by osteoclastic bone resorption, activated by humoral factors that are released from tumor or stromal cells. Recently, Integrin αβ3 has been shown to play an important role in the attachment of osteoclasts to bone matrix (9), and the stimulation of Integrin αβ3 expression by human osteoclasts stimulates adhesion and migration in the bone matrix (10). Currently, Integrin αβ3 antagonists are being developed as inhibitors of bone resorption (11). On the other hand, breast cancers commonly cause osteolytic metastasis according to the type of interaction between breast cancer cells and osteoblasts and osteoclasts (3). Breast cancer cells residing in bone overexpress Integrin αβ3, suggesting either a subclonal selection of Integrin αβ3-expressing tumor cell populations or up-regulation of Integrin αβ3 in the bone microenvironment. We assumed that, if breast cancer cells overexpressed Integrin αβ3, they are more likely to adhere to bone matrix and interact with osteoblasts and osteoclasts.

We studied the expression of Integrin αβ3 in four human breast cancer cell lines in various ways. Integrin αβ3 was expressed in two human breast cancer cell lines, MDA-MB-231 and MDA-MB-435, but not in MKL-4 or T-47D. This result is consistent with a previous report (12). Thus, it is clear that different human breast cancer cell lines differ in their expression of Integrin αβ3, though all human breast cancer cell lines expressed Integrin αβ1. Integrin αβ1 is a collagen receptor which is commonly expressed in human breast cancer and its loss has been reported to be associated with the invasive phenotype of breast cancer (13). However, in the present study, Integrin αβ1 was not related to BM.

Our attempts to create a model of BM using MDA-MB-231 and 435 cells with Integrin αβ3 were successful. We considered the seed and soil theory consistent with our data (14), i.e., both cells are likely to adhere to bone matrix only when the bone marrow environment had the ligand for Integrin αβ3, osteopontin.

It is reasonable to question the homology between human and rat cDNA for Integrin αβ3. Dolan et al. (15) reported that rat cDNA fragments shared 86 to 91% homology with their respective human sequences. So, at least theoretically, using human cDNA in this rat model is justified.

We successfully developed BM by inoculating rats with MDA-MB-231 [9/11 (81.8%)] and MDA-MB-435 cells [8/10 (80.0%)], both of which express Integrin αβ3. On the other hand, we failed to consistently develop BM using MKL-4 cells which lack Integrin αβ3 [1/9 (11.0%)]. This result shows that expression of Integrin αβ3 by breast cancer cells is critical to the formation of BM.

We have shown that the dis-integrin echistatin, arg-gly-asp (RGD)-containing peptide, blocks migration of
Table I. Expression of Integrins $\alpha_\nu\beta_3$ and $\alpha_2\beta_1$ in breast cancer cell lines.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>$\alpha_\nu\beta_3$</th>
<th>$\alpha_2\beta_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MDA-MB-435</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MKL-4</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>T-47D</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Table II. Frequency of bone metastasis (BM) after injection of breast cancer cell lines.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Frequency of BM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>9/11 (81.8)</td>
</tr>
<tr>
<td>MDA-MB-435</td>
<td>8/10 (80.0)</td>
</tr>
<tr>
<td>MKL-4</td>
<td>1/9 (11.0)</td>
</tr>
</tbody>
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Figure 2. Overexpression of Integrin $\alpha_\nu\beta_3$ demonstrated immunohistochemically in A: MDA-MB-231 and B: MKL-4.

Figure 3. $\alpha_\nu$ and $\beta_3$ subunit mRNA in human breast cancer cell lines.

The details of the mechanism by which Integrin αvβ3 is related to BM are unclear. We do not know whether activation of Integrin αvβ3 in human breast cancer cells stimulates adhesion or migration in response to osteopontin in vivo, as it does in the osteoclast. The breast cancer cells that express Integrin αvβ3 clearly acquire the ability to adhere to the bone matrix. Further study of Integrin αvβ3's role in the development of BM from human breast cancer is required.

Conclusion

1) Integrin αvβ3 is expressed in the MDA-MB-231 and MDA-MB-435 human breast cancer cell lines, but not in MKL-4 or T47D.
2) MDA-MB-231 and MDA-MB-435 are more likely to cause bone metastasis in rats than MKL-4.

References


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