Motility Related Protein 1 (MRP-1/CD9) Expression: Inverse Correlation with Metastases in Breast Cancer

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ABSTRACT

In our previous studies we showed that motility related protein 1 (MRP-1) is a glycoprotein recognized by mAb M31-15, and that the sequence of MRP-1 is identical to that of CD9, a WBC differentiation antigen. Transfection of MRP-1/CD9 cDNA into cultured nonhematopoietic cells suppresses cell motility. The extent of suppression is directly related to the level of MRP-1/CD9 expression. In addition, the metastatic potential of MRP-1/CD9-transfected melanoma BL6 cells is lower than that of control BL6 cells. To determine whether these experimental results are of relevance with respect to actual human tumors, we investigated MRP-1/CD9 expression in 143 invasive ductal carcinomas of the breast. Of 97 patients with MRP-1/CD9-positive tumors, only 36 (37.1%) had lymph node involvement. In contrast, 21 of 39 (53.8%) patients whose tumors had reduced MRP-1/CD9 immunoreactivity and 5 of 7 patients whose primary carcinomas were not stained by the anti-MRP-1/CD9 MAb had lymph node metastases. The comparison of protein expression by 62 primary tumors and their respective metastatic lymph nodes revealed that in almost 50% of the cases, the latter had lower MRP-1/CD9 levels than the former. Moreover, reverse transcriptase-PCR-based analysis disclosed that MRP-1/CD9 gene expression in the metastatic lymph nodes of 17 of 32 patients was strikingly lower than in the primary invasive ductal carcinomas. Gene overexpression was not observed in any of the samples studied. Our data suggest that low MRP-1/CD9 expression may be associated with the metastatic potential of certain human tumors.

INTRODUCTION

Cell motility is one of the rather essential complex functions operative in inflammation, tissue repair, angiogenesis, and tumor invasion (1). We have shown previously that motility of MAC10 cells, derived from a human adenocarcinoma of the lung, is inhibited by mAb M31-15, and that this antibody recognizes MRP-1, a transmembrane glycoprotein (2). We also showed that the sequence of MRP-1 cDNA is identical to that of CD9 (2), reported in the same year by two other groups (3, 4). CD9 is a M, 24,000-27,000 glycoprotein that is widely expressed on hematopoietic tissues, platelets, eosinophils, basophils, stimulated T lymphocytes, and early B cells, but not on resting T and B cells (3–5). Several studies have demonstrated that mAbs to CD9 cause platelet activation and aggregation by a mechanism similar to that of thrombin (3–6). Moreover, it has been suggested that this type of platelet activation is coupled to phospholipase A2 and to phospholipase C stimulation, which in turn induces an increase in cytoplasmic calcium and protein tyrosine phosphorylation (7–9), thus pointing to the direct participation of CD9 in signal transduction. Although we found that MRP-1/CD9 is expressed on most cell lines derived from solid human tumors (2), the functions of MRP-1/CD9 in non-hematopoietic cells are still unknown. In an effort to clarify the functions of MRP-1/CD9 in such cells, we transfected various types of cultured cells with plasmid constructs containing MRP-1/CD9 cDNA (10). These experiments revealed that cell motility and growth were suppressed in the MRP-1/CD9-expressing cells. In addition, studies with the mouse melanoma BL6 cells BALB/c-nu/nu mouse system (11) disclosed that the metastatic potential of all transformants expressing MRP-1/CD9 was lower than that of the parent BL6 cells. These findings would suggest that MRP-1/CD9 regulates cell motility and is a receptor for negative signal ligands. On the basis of these experimental results, we investigated whether there is a relationship between primary human tumors and their metastases with respect to MRP-1/CD9 expression. In this report we present the results of such a study, carried out on surgically resected breast cancer tissues and the respective metastatic lymph nodes.

MATERIALS AND METHODS

Patients and Surgical Specimens. We studied specimens from 143 patients with invasive ductal carcinoma of the breast who underwent surgery at the Department of Thoracic Surgery of the Kitano Hospital, Medical Research Institute of Osaka between May 1990 and December 1992. Ten patients with intraductal carcinoma and nine with double cancers were not included in the study. Four patients with distant metastases were also excluded from this study. The salient clinical characteristics of the patients are presented in Table 1. One-half of each fresh tumor tissue and each lymph node obtained at surgery were immediately embedded in optimum cutting temperature compound (Miles, Elkhart, IN) and frozen at −80°C. Frozen sections were cut on the cryostat to a thickness of 6 μm, mounted on poly-L-lysine-coated slides, and either used immediately or stored at −80°C until needed. In each case, one additional section was stained with hematoxylin and eosin for verifying the presence of cancer cells, especially in the metastatic lymph nodes. One-half of a given lymph node specimen containing only cancer cells was used for Western blotting and RT-PCR.

Immunohistochemical Assays. Because the MRP-1/CD9 antigen is not preserved in formalin-fixed, paraffin-embedded tissues, we used frozen sections for these assays; they were performed as described previously (2, 12). Briefly, the sections were immersed for 30 min in 0.3% H2O2 (in absolute methanol) to inhibit endogenous peroxidase activity and then treated with 5% BSA. Sections were incubated for 2 h with the anti-MRP-1/CD9 MAb M31-15, sequentially followed by incubation with biotinylated horse antihorse IgG and the avidin-biotin-peroxidase complex. Antibody binding was visualized with 3,3′-diaminobenzidine tetrahydrochloride in the presence of 0.05% H2O2. The sections were then counterstained with hematoxylin, dehydrated, and mounted. Biotinylated horse antihorse IgG, avidin, and biotin were purchased from Vector Laboratories, Inc. (Burlingame, CA). Sections incubated with mouse myeloma SP2 supernatant served as negative reaction controls.

Western Blotting. Cancer cell-containing tissue samples were selected and solubilized with 1% CHAPS. An aliquot of the soluble fraction was subjected to slab gel electrophoresis, followed by Western blotting and probing with M31-15 mAb, rabbit antitissue IgG (γ-chain specific; Cappel, Malvern, PA) and 125I-protein A (DuPont New England Nuclear, Boston, MA; Refs. 13, 14). The total cellular protein content applied to each lane was adjusted to identical concentrations. Cells of the human breast cancer line ZR-75-30 were used as positive controls. Band intensity was evaluated by densitometry.
Specimen Classification Based on Immunohistochemical and Western Blotting Results. When >50% of the carcinoma cells in a given specimen were positively stained, the sample was classified as MRP-1/CD9 positive (+); when 5–50% were stained, as reduced (±); and when <5% were stained, as negative (−). To classify the specimens on the basis of Western blotting, the Mr 25,000 band obtained with the control cells ZR-75-30 was set at 100%. Patient samples with a band intensity of >30% were classified as MRP-1/CD9 positive (+), those with intensities between 3 and 30% as reduced (±), and <3% as negative (−).

RT-PCR Analysis. Total cellular RNA was isolated from fresh or frozen tumor tissues by the acid guanidinium thiocyanate procedure (15). Randomly primed cDNA was prepared from 5 μg of total RNA by using Maloney murine leukemia virus RT (Pharmacia, Piscataway, NJ) and following the manufacturer’s protocol. One μl aliquot of the reaction was used for PCR amplification. MRP-1/CD9-specific oligonucleotides synthesized on the basis of the nucleotide sequence (2–4) served as primers; 5′-TGCATCTGTATCAGCGCCA-3′ was the sense primer, and 5′-CTCAGGGATGTAAGCTGACT-3′ was the antisense primer. β-actin amplification was used as internal
MRP-1/CD9 EXPRESSION IN BREAST CANCER

Fig. 2. Immunoblot analysis of MRP-1/CD9 expression in breast cancer. Lane 1, breast cancer cell line ZR-75-30 (positive control); Lane 2, MRP-1/CD9-positive primary invasive ductal carcinoma; Lane 3, primary invasive ductal carcinoma with reduced MRP-1/CD9 levels; Lane 4, invasive ductal carcinoma with negative MRP-1/CD9; Lanes 5, 7, 9, and 11, primary invasive carcinomas; Lanes 6, 8, 10, and 12, metastatic lymph nodes of these four carcinomas. Note that intensities of the lymph node bands are less than those of the respective primary breast tumors. K, molecular weight in thousands.

PCR control (16); the sense primer was 5'-CTGTCCTGGCGGACCCACCAT-3', and the antisense primer was 5'-GCAAATAAGCTAGTTTCCGC-3'. The MRP-1/CD9 primer pair amplifies an 800-bp fragment (nucleotides 19–818) that includes the full-length cDNA. Forty PCR amplification cycles were carried out in a thermal cycler, each consisting of 40 s at 94°C, 40 s at 60°C, and 90 s at 72°C. The same conditions were used to amplify β-actin DNA. Tubes containing all ingredients except templates were included in all runs as negative reaction controls. The amplified DNA samples were subjected to agarose (1%) gel electrophoresis; bands were visualized with ethidium bromide and photographed with a Polaroid camera. Densitometric analysis of the photographic negatives was used for band quantification (17). The values obtained for MRP-1/CD9 in a given sample were divided by that of β-actin and referred to as MRP-1/CD9 expression ratio. The expression ratio of a metastatic lymph node was divided by that of its corresponding primary tumor to obtain the MRP-1/CD9 rate of reduction. Final values ranging from 0.5 to 2.0 were considered as indicating no reduction in gene expression, and values of <0.5 as denoting a decrease.

RESULTS

Immunohistochemical Detection of MRP-1/CD9 in Normal, Fibroadenoma, and Cancer Tissues of the Breast. As shown in Fig. 1A normal mammary ducts and glands were intensely stained by the anti-MRP-1/CD9 antibody. Without exception, all epithelial cells expressed the protein. On the other hand, there was a striking difference between benign fibroadenomas and breast cancer tissues. The specimens from all 23 cases of fibroadenoma were strongly stained (Fig. 1B); almost all benign tumor cells expressed MRP-1/CD9. The reaction product deposits were located at the cell membrane. In contrast, various distinct staining patterns were noted with the cancer tissues. Of the 143 primary breast cancers studied, 97 cases (67.8%) were evaluated as MRP-1/CD9 positive. In these cases, expression resembled that of normal glands and benign fibroadenomas because immunostaining was intense and seen uniformly at the surface membrane (Fig. 1C). There were 39 cases (27.2%) with reduced (±) MRP-1/CD9 expression (Fig. 1D). In most of these tumors, expression was heterogeneous, and the immunostaining pattern along cell junctions was not linear, but rather granular. No MRP-1/CD9 immunoreactivity was observed with the specimens of 7 (4.9%) of the 143 breast cancer patients examined. An example of such a case is shown in Fig. 1E.

Immunoblot Analysis. The major M, 25,000 MRP-1/CD9 band was clearly evident in the Western blots of the soluble fraction of the primary tumors that were positively stained in the immunohistochemical assays (Fig. 2). In contrast, the band intensity was weak or absent in the primary invasive ductal carcinomas that had reduced or no immunohistochemically detectable MRP-1/CD9. Overall, the immunoblotting results agreed well with those of immunohistochemical staining, and in case of discrepancy, the results of Western blotting were used in specimen classification.

Relationship between Immunodetected MRP-1/CD9 and Known Prognostic Factors. As shown in Table 1, we found no statistically significant relationship (χ²) between protein expression and the age of patients at surgery, estrogen and progesterone receptor status, or tumor size. In contrast, there was a significant relationship between MRP-1/CD9 expression and lymph node status (P = 0.0045).

Comparison of Immunohistochemical Staining Patterns of Primary Tumors and Their Respective Metastatic Lymph Nodes. The analysis of the 62 cases from whom primary tumors and lymph node metastases were studied revealed that immunostaining intensity of the lymph nodes of all patients was equal or lower but never higher than that of the corresponding primary carcinoma (Table 2). Of the 36 patients whose primary tumors had high protein levels, expression in the involved lymph nodes was at the same level in 22 and lower in 14 (Fig. 1F). Moreover, the metastatic lymph nodes of 9 of 21 patients whose primary carcinoma had reduced protein levels were classified as MRP-1/CD9 negative (Table 2).

RT-PCR Analysis of the MRP-1/CD9 Gene in Primary Tumors and Their Metastatic Lymph Nodes. Lymph nodes with normal lymphocytes were excluded from RT-PCR analysis. We found no evidence of PCR-mediated MRP-1/CD9 gene amplification in primary tumors and their respective lymph node metastases of patients whose breast carcinoma cells were not positively stained by the anti-MRP-1/CD9 antibody. These observations are in accord with our recent experimental findings that the levels of the MRP-1/CD9 gene product depend on the quantity of transfected cDNA (10). On the other hand, a comparison between protein-positive tumors and their respective lymph nodes revealed that the band intensities of the amplified MRP-1/CD9 DNAs were strikingly greater in the primary tumor specimens than in the metastatic lymph nodes (Fig. 3). We
found that the amount of MRP-1/CD9 cDNA was reduced (rate, <0.5) in the lymph nodes of 17 of 32 (53.1%) of the patients whose specimen pairs were subjected to RT-PCR analysis. This set of observations provided corroboration at the cDNA level of the results obtained in the immunohistochemical assays. Overexpression of the MRP-1/CD9 gene was not seen with either type of specimen. Amplification of the β-actin gene, used as an internal PCR control, was observed in all instances (Fig. 3).

**DISCUSSION**

Several cDNAs encoding for human MRP-1/CD9 have been isolated (3, 4). From the characterization of the clones and homology searches it was determined that MRP-1/CD9 belongs to the TM4SF, which comprises 15 members that are variously expressed on leukocytes, a variety of mammalian tissues, and even on the surface of two types of parasites (5, 19). The precise biochemical functions of the TM4SF are not known, but current data largely suggest that they are involved in the regulation of cell development, proliferation, activation, and motility (5, 19). The melanoma-associated antigen ME491/CD63 is an interesting molecule that also belongs to the TM4SF. It is strongly expressed in dysplastic nevi and radial growth-phase primary melanomas, but expression declines significantly, and sometimes disappears entirely, as the melanoma cells progress to more malignant stages such as vertical growth-phase primary melanoma and metastatic melanoma (20). On the other hand, an examination of the relationship between metastatic potential and other TM4SF molecules, including CD37, CD53, ME491/CD63, TAPA-1/CD81, as well as MRP-1/CD9 suggested that only the latter was expressed predominantly by primary, rather than by metastatic tumors (21). These findings are very consistent with our data that MRP-1/CD9 expression diminishes as the clinical stage of a given breast cancer advances. In addition, recently it was reported that an exciting gene (KAI-1) was a metastasis suppressor gene for prostate cancer. At least MRP-1/CD9, ME491/CD63, and KAI-1 among TM4SF may play important roles in tumor progression and metastasis (22). Furthermore, because the disruption of cell-to-cell contacts induces metastasis of tumor cells, it is of interest in the present context that anti-CD9 mAbs elicit an enhancement of Fc-independent heterotypic adhesion of pre-B-cell lines to bone marrow stromal fibroblasts but not to bone marrow stroma (6). Thus, on the basis of our previous experimental finding that the anti-MRP-1/CD9 mAb inhibits cell motility of various types of cancer cells (1, 2), it is conceivable that MRP-1/CD9 affects motility and invasiveness by modulating the level of cellular adhesion.

Various types of human carcinomas were recently examined for the presence of E-cadherin, a member of the family of calcium-dependent cell adhesion molecules (23). It was shown that E-cadherin immunoreactivity was inversely related to tumor differentiation and metastasis (24, 25), and that mutations of E-cadherin gene may contribute to the development of the diffuse type of gastric carcinomas (18). It is conceivable that this may also apply to MRP-1/CD9 because we found a significant relationship between MRP-1/CD9 immunoreactivity and lymph node status, as well as lower protein and MRP-1/CD9 mRNA levels in the metastatic lymph nodes than in the respective primary carcinomas of around 50% of the patients. These observations suggest that low expression of the MRP-1/CD9 gene may be a feature of some breast tumors with high metastatic potential.

However, it must be noted that there were certain patients with lymph node metastases whose primary tumors were immunohistochemically MRP-1/CD9 positive, even at an early stage. These differences with respect to MRP-1/CD9 expression point to several possible explanations: (a) the anti-MRP-1/CD9 mAb M31-15 cannot discriminate between a normal and a mutant protein; (b) a gene

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**Table 1 Relationship of MRP-1/CD9 immunostaining and various prognostic factors**

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<td>8 (10%)</td>
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<td>12 (10.4%)</td>
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<td>143</td>
<td>97</td>
<td>39</td>
<td>7</td>
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*a*, positive immunostaining; ±, reduced expression; −, negative immunostaining.

**Table 2 Relationship between MRP-1/CD9 expression in primary tumors and in metastatic lymph nodes (immunohistochemical assay)**

<table>
<thead>
<tr>
<th>MRP-1/CD9 expression in primary tumor</th>
<th>MRP-1/CD9 expression in metastatic lymph node</th>
<th>Total</th>
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<td>(+)</td>
<td>(+)</td>
<td>22 (61.1%)</td>
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<td>(±)</td>
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<td>(−)</td>
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<tr>
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<td></td>
<td>22</td>
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*a*, positive immunostaining; ±, reduced expression; −, negative immunostaining.
mutation could give rise to an altered protein with diminished normal function; or (c) because MRP-1/CD9 has one N-linked and several O-linked glycosylation sites, an aberrant glycosylation could result in loss of the protein's function. These and other questions about the role, if any, of MRP-1/CD9 in solid tumors remain to be answered in the near future. Although it has not yet been determined that MRP-1/CD9 is a functional molecule, our results suggest that its diminished expression appears to be associated with metastases, and that the protein may represent a potentially useful prognostic marker. However, large-scale prospective and retrospective studies on various types of cancers are needed to establish whether MRP-1/CD9 is indeed of practical utility as a prognostic predictor.

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