Identification of a Cell-Surface Antigen (LEA.135) Associated with Favorable Prognosis in Human Breast Cancer

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Abstract

The present study was undertaken with a rationale that loss of certain "normal tissue" antigens might have prognostic significance, reflecting inactivation of the corresponding genes during neoplastic progression. An attempt was made to identify such antigens by means of generating monoclonal antibodies using a tolerization/immunization procedure. A monoclonal antibody generated by immunization of BALB/c mice with normal breast tissue extract, following prior tolerization with mammary carcinoma cells, recognized a cell-surface glycoprotein, luminal epithelial antigen, with an apparent molecular weight of 135,000 (LEA.135). The pattern of expression on LEA.135 was determined by immunohistochemical-staining techniques on frozen and formalin-fixed and paraaffin-embedded tissue sections. LEA.135 was demonstrable on the apical plasma membrane of normal and nonneoplastic epithelial cells in breast and other tissues. Studies have shown that LEA.135 is distinct from receptors for epidermal growth factor and from known antigens associated with epithelial cells, including the family of keratins. In a retrospective study, with a follow-up ranging from 5 to 15 years, patients whose breast tumor cells expressed LEA.135 had a superior overall survival rate (78% 0.139% at >5 years; P = 0.025). Furthermore, in patients with histologically poorly differentiated tumors, LEA.135-positive cases had a better prognosis (80 0.179% at >5 years; P = 0.013) compared with LEA.135-negative cases. In addition, in patients with aneuploid tumors, LEA.135-positive cases again showed an improved survival (90 0.001% at >5 years; P = 0.039) compared with those that were with LEA.135 negative. The results suggest that the expression of LEA.135 provides a useful indication of clinical outcome in patients with breast carcinomas.

Introduction

Loss of heterozygosity for genes on chromosome 1q, 11p, 13q, and 17p in human mammary carcinoma cells has been documented (1-5). However, the reduction to homo- or hemizygosity has not been identified at a molecular level. In the current study, an attempt was made to identify any products of gene(s) that may become inactivated in invasive carcinoma cells. In order to achieve the stated goal, the procedure of tolerization/immunization (6) was modified to favor the generation of antibodies to cell products associated with normal cells but lacking malignant cells. The tolerance to invasive malignant mammary epithelial cell lines (MCF-7 and MDA-MB-231) was induced in neonatal mice, prior to subsequent immunization with an extract of normal breast tissue. A monoclonal antibody that exhibited binding activity with a cell-surface glycoprotein present on normal breast cells, but absent in certain cases of primary breast carcinomas, was identified. The corresponding antigen was purified and termed LEA.135.

Based on the above observation, a retrospective investigation was carried out to investigate the possible prognostic value of LEA.135 expression in 40 cases of primary breast carcinomas with 5-15 years of follow-up. The results suggest that patients whose primary tumor cells express LEA.135 have a significantly favorable prognosis.

Materials and Methods

Comparison of Epitopes. Competitive immunocytochemically steric-inference assays were performed using immunocytochemical techniques in order to compare the nature of the epitopes recognized by anti-LEA.135 antibody to those reacting with previously reported antibodies to human mammary epithelial cells. Sections of breast tissue containing normal epithelial cells were incubated first with the unlabeled test antibodies that included epithelial membrane antigen (7), MFGM-gp70 (8, 9), MFGM-gp155 (10, 11), HMFG-1 (12), HMFG-2 (13), pan keratin (14), and receptor for epidermal growth factor (15), followed by incubation with predetermined concentration of biotinylated anti-LEA.135 antibody. The remainder of the staining procedure was as described previously (6). Any change in the intensity of staining with reference to control preparations was recorded.

Metabolic Labeling of Cells and Preparation of Cell Lysate. Owing to the absence of anti-LEA.135 antibody's reactivity to the established tumorigenic mammary carcinoma cell lines (e.g., MCF-7, MDA-MB-231, ZR-75.1, HSS787), a model system that consists of nontumorigenic immortalized MEC lines was utilized in this study (16-18). The cell lines designated 184A1 and 184B5 were grown as described by Stampfer (17). Briefly, MEC lines were grown as monolayer cultures in 75-mm² tissue culture flasks and intrinsically labeled when cultures were still subconfluent. The cells were labeled for 24 to 48 h with either 2 mCi of [3H]leucine or galactosamine (110 Ci/mmol) per flask of leucine or galactosamine-free Dulbecco’s minimum essential medium, respectively. Following incubation, the cells were washed 3 times and lysed with 0.05 M Tris-HCI buffer, pH 7.5, containing 0.15 M NaCl, 0.5% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, I ITIM phenylmethylsulphonyl fluoride, and 0.5 mM chloromethyl-ç-(2-phenyl-1-p-toluenesulphamoyl)ethyl ketone on ice for 15 min. The lysates were centrifuged at 40,000 × g and 4°C for 10 min. The supernatant containing detergent-solubilized materials were subsequently used for immunoprecipitation.

Comparison of LEA.135 with Other Antigens Associated with MEC. Competitive immunoprecipitation analyses were performed to ascertain the nature of antigen recognized by anti-LEA.135 antibody in relation to the antigens of other epithelial cells (7-15). Prior to immunoprecipitation of the various cell line extract (see metabolic labeling), each sample (1 mg protein/ml) was first preabsorbed separately with antibody to LEA.135, epithelial membrane antigen, MFGM-gp70, MFGM-gp155, HMFG-1, HMFG-2, pan keratin, or receptor for epidermal growth factor, immobilized individually to Sepharose 4B (5 m antibody/ml of Sepharose 4B) as described previously (19-21). Preabsorption was carried out by mixing and incubating the suspension overnight at 4°C. Following an overnight incubation, the suspension was centrifuged at 10,000 × g and 4°C for 15 min. The supernatant containing antibody-preabsorbed extract was then subjected to immunoprecipitation with each antibody separately and analyzed by sodium dodecyl sulfate-polycrylamide gel electrophoresis and fluorography as described previously (19-21). The negative control consisted of the extract which was preabsorbed with the antibody that subsequently also served for immunoprecipitation.

Patients. Tissue sections were obtained from the University of California (Davis) Medical Center, Sacramento, CA. The following data were obtained by

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2. To whom requests for reprints should be addressed.

3. The abbreviations used are: LEA.135, luminal epithelial antigen with an apparent Mr, 135,000; gp, glycoprotein; MFGM, milk fat globule membrane; HFMG, human milk-fat globule; MEC, mammary epithelial cells; OS, overall survival.

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chart review of the corresponding patients: age and tumor stage at the time of biopsy; quantitative estrogen receptors status; nodal status; histological subtype; time to progression in years (defined as tumor recurrence, progression, or cancer-related death); or OS.

**Histological Evaluation.** Tissue sections, stained with haematoxylin and eosin were evaluated independently of the immunohistologic staining for histopathological type, nuclear grade and degree of morphological differentiation. Immunostained sections were reviewed independently by three pathologists, (C. R. T., R. D. C., and R. S. C.), who were blinded to status of patients. By prior agreement, cases with 10% or more cells showing surface and/or cytoplasmic reactivity were deemed positive. Heterogeneity in the intensity of staining of tumor cells within samples, noted in a minority of cases, was not included in the determination of the score.

**Statistical Analysis.** Initially, univariate analyses were performed where the association with OS was examined for each of the following prognostic factors: morphological differentiation; age, ploidy; axillary lymph node involvement; and estrogen receptor status. For each of these factors, a Kaplan-Meier curve was drawn and a log-rank test was performed. To determine whether LEA.135 expression independently contributed significant prognostic information, above and beyond these factors, a stratified log-rank test was performed, with OS as the outcome and LEA.135 expression as the predictor of interest, and morphological differentiation, age, ploidy, axillary lymph node involvement, and estrogen receptor status as the stratification variables.

Preparation and Staining of Tissue Sections. Normal and neoplastic human tissues were obtained from the surgical pathology files of the University of California Medical Center. Tissue used were fixed in formalin. Tissues were sectioned at 5 μm and representative sections were stained with haematoxylin and eosin to confirm the diagnosis prior to immunostaining. Staining observed in frozen and formalin-fixed, paraffin-embedded sections was similar quantitatively and qualitatively, leading to a preference in the latter, because of superior morphology and also for performing retrospective studies. One hundred μl of the antibody (1 μg/ml) were applied directly to tissue sections and localized by an immunoperoxidase method as described previously (6). The primary antibody, preabsorbed with the immunogen (an extract of normal breast tissue), served as a negative control. Histological classification of breast cancer tissue was determined according to the criteria of Bloom and Richardson (22).

**Results**

**Comparison of Epitope.** Comparison was made between epitopes recognized by anti-LEA.135 antibody with those of the previously described in relation to mammary epithelial cells (8–16). The immunoblocking assays showed that the antigenic binding site for anti-LEA.135 antibody was not blocked by other antibodies, suggesting that the epitope recognized by anti-LEA.135 antibody is distinct (Table 1). Furthermore, the component recognized by anti-LEA.135 antibody is also different with respect to its molecular weight (results not shown).

**Comparison of LEA.135 with the Antigens of Other Epithelial Cells.** LEA.135 was compared with the antigens of other known epithelial cells by competitive immunoprecipitation method. The Nonidet P-40 solubilized extracts of metabolically labeled immortalized MEC lines (184A1 or 184B5) were used as sources of the target antigens for all eight antibodies included in this study. Each of the antibodies immunoprecipitated their corresponding antigen, and when used for preabsorption they inhibited specific precipitation, as revealed by fluorography. Preabsorption of extract against each of the other seven antibodies did not diminish recovery of LEA.135, whereas absorption with anti-LEA.135 antibody removed all traces of precipitate (Fig. 1). For example, antibodies to LEA.135 and MFGM-gp155 exhibited a strong reactivity with their corresponding antigens with the apparent molecular weights of 135,000 and 155,000, respectively (Fig. 1, Lanes B and F). The application of immunoprecipitant, resulting from incubation of anti-LEA.135 antibody and the radiolabeled cell lysate of the immortalized MEC line (184A1) that was preabsorbed with immobilized anti-MFGM-gp155 antibody, exhibited a band corresponding to LEA.135 (Fig. 1, Lane C). Likewise, preabsorption of the extract with the immobilized anti-LEA.135 antibody showed no effect on the antigen recognized by anti-MFGM-gp155 antibody (results not shown). The incubation of the lysate (184A1), which was preabsorbed and subsequently incubated with the same antibody, failed to immunoprecipitate the corresponding antigens (Lanes D and E). Molecular weight standards are shown in Lane A.

**Immunohistological Localization of LEA.135.** In normal breast tissues, LEA.135 was expressed predominantly on the apical plasma membrane of luminal epithelial cells lining the ducts (Fig. 2). In benign breast diseases, such as fibroadenoma or hyperplasia, expression of LEA.135 was exhibited much as in normal breast. In a retrospective study of 40 cases of primary breast carcinomas, expression of LEA.135 varied from case to case: some tumors were entirely lacking in positivity, while other examples showed distinct membrane and/or surface positivity of 10–100% of tumor cells. Those patients with tumors displaying 10% or more positive cells independently correlated with a favorable prognosis (Fig. 3). A comparison of OS was made of patients whose tumor cells exhibited immunoreactivity with anti-LEA.135 antibody compared with those whose specimens showed absence of LEA.135 expression. A statistically significant

### Table 1 Comparison of epitopes detected by anti-LEA.135 and other known antibodies to epithelial antigens by immunohistochemical steric-inference (blocking) assay

<table>
<thead>
<tr>
<th>Initial incubation* (antibody to)</th>
<th>Second incubation</th>
<th>Immunoperoxidase staining</th>
<th>Intensity of staining*</th>
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<tr>
<td>PBS</td>
<td>Biotinylated anti-LEA.135</td>
<td>ABC</td>
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<td>EMA</td>
<td>Biotinylated anti-LEA.135</td>
<td>ABC</td>
<td>+3</td>
</tr>
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<td>ABC</td>
<td>+3</td>
</tr>
<tr>
<td>EGF-R</td>
<td>Biotinylated anti-LEA.135</td>
<td>ABC</td>
<td>+3</td>
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* Positive controls to demonstrate effective binding of antibody to MFGM-gp70, MFGM-gp155, EMA, HMFG-1, HMFG-2, keratin, and epidermal growth factor receptor were performed using the indirect immunoperoxidase method and the appropriate tissue sections and confirmed that each of these antibodies bound to target cells.

* Mean value for three different tissue sections utilized in this study.

* PBS, phosphate-buffered saline; EMA, epithelial membrane antigen; ABC, Avidin-biotin-peroxidase complex; EGF-R, epidermal growth factor receptor.

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**Fig. 1.** Comparison of antigens recognized by antibodies to LEA.135 and MFGM-gp155. LEA.135 was compared with other known epithelial antigens, such as MFGM-gp155, by competitive immunoprecipitation method as described in the text. Antibodies to LEA.135 and MFGM-gp155 exhibited reactivity with their corresponding antigen with an apparent molecular weight of 135,000 and 155,000, respectively (Lanes B and F). The application of immunoprecipitant, resulting from incubation of anti-LEA.135 antibody and the radiolabeled cell lysate of the immortalized MEC line (184A1) that was preabsorbed with immobilized anti-MFGM-gp155 antibody, exhibited a band corresponding to LEA.135 (Lane C). The incubation of lysate (184A1) which was preabsorbed and subsequently incubated with the same antibody failed to immunoprecipitate their corresponding antigens (Lanes D and E). Molecular weight standards are shown in Lane A.
Fig. 2. Reactivity of anti LEA.135 antibody with malignant mammary epithelial cells in paraffin-fixed tissue sections by an indirect immunohistological staining method. Malignant mammary epithelial cells from a patient with good prognosis (survival >5 yrs). The antibody shows reactivity heterogeneously with the malignant cells. The connective tissue cells were consistently negative. The sections were counterstained with Mayer's haematoxylin (X 312).

Fig. 3. Kaplan-Meier plot of disease progression in patients whose tumor cells were LEA.135 positive (A: score = 2; n = 9) versus LEA.135 negative (B: score = 0, n = 31). Time was measured from biopsy to last follow-up in year.

Fig. 4. Kaplan-Meier plot of disease progression in patients whose tumor cells were morphologically poorly differentiated and LEA.135 positive (A: score = 2; n = 5) versus LEA.135 negative (B: score = 0; n = 15). Time was measured from biopsy to last follow-up in year.

Fig. 5. Kaplan-Meier plot of disease progression in patients whose tumor cells were aneuploid and LEA.135 positive (A: score = 2; n = 3) versus LEA.135 negative (B: score = 0; n = 14). Time was measured from biopsy to last follow-up in year.

univariate association between LEA.135 expression and OS was observed (log-rank, P = 0.025).

Furthermore, among patients with histologically poor differentiated tumors, LEA.135-positive cases had an improved survival (80 ± 0.179% at >5 years; P = 0.013) compared with those that were LEA.135-negative (67 ± 0.064% at >5 years) (Fig. 4). Likewise, patients with aneuploid tumors, who were LEA.135 positive again exhibited a better prognosis (90 ± 0.001% at >5 years; P = 0.039) compared with those who were LEA.135 negative (21 ± 0.110% at >5 years) (Fig. 5). Interestingly, a subset of patients with well differentiated, diploid tumor cells and/or uninvolved axillary lymph nodes who were LEA.135 positive and showed a trend, although statistically insignificant, of an improved overall survival for more than 5 years compared with those who were LEA.135 negative.

Discussion

Selection of the appropriate overall therapy of patients with breast cancer remains uncertain. Many patients show lasting benefit; others do not, unpredictably so. For these reasons, the cellular and molecular changes leading to mammary carcinomas warrant study, with a view to identification of patients at particular risk, patients likely to benefit from various types of adjunctive therapy.

In the present study, an approach of tolerization with breast tumor cells and immunization with their normal counterparts was taken in an attempt to develop monoclonal antibodies with specificity for antigens present on normal breast epithelium but absent in the invasive and metastatic mammary carcinoma cells. During the initial screening by immunohistological methods, freshly frozen sections of breast tissue were used. An antibody that reacted to an antigen having the above properties was selected for further study. Staining observed in frozen or formalin-fixed tissue sections was similar both quantitatively and qualitatively, leading to a preference for the latter for retrospective studies. The antigen recognized by the antibody was luminal epithelial antigen. With an apparent molecular weight of 135,000, this antigen (LEA.135) appears to be distinct from other known antigens associated with epithelial cells (7-16), a conclusion supported by competitive blocking (Table 1) and immunoprecipitations studies (Fig. 1).

Competitive immunoprecipitation experiments were performed to determine whether the reactivity of anti-LEA.135 antibody differed significantly from the antibodies of the antigens of other antiepithelial cells which are commercially available or described in the literature. Taken in conjunction with specificity and molecular weight data, these clearly distinguish anti-LEA.135 antibody from all of the seven antibodies to the antigens of epithelial cells included in this study (8-16). Furthermore, anti-LEA.135 antibody was nonreactive with all the mammary carcinoma cell lines included in this study, whereas all the seven test antibodies showed a variable reactivity with these cell lines.

In normal breast tissue as well as in benign breast diseases such fibroadenoma and hyperplasia, epithelial cells exhibited strong expression of LEA.135. A similar pattern of expression of LEA.135 was observed in normal extramammary glandular tissues such as endometrium, colon, lung, pancreas, and stomach. In primary mammary carcinomas, LEA.135 was not detected in some of the cases but was present on others, irrespective of the morphological appearance of the
tumor cells. Such an observation prompted a retrospective study of mammary carcinomas, in which known survival data allowed separating into two groups, with both good or poor prognosis. In this double-blind retrospective study, expression of LEA.135 was predictive of OS for patients with primary breast carcinomas. The predictive prognostic-value of LEA.135 expression was statistically significant, as demonstrated in the present study. The results suggest that expression, or lack thereof, of LEA.135 may identify subgroups of patients who have particularly favorable or unfavorable prognosis. That this discrimination was particularly evident in poorly differentiated tumors and in patients with aneuploid tumor cells is consistent with the working hypothesis of progressive loss of the antigen in advanced malignancy. Whether these findings warrant different treatment strategies deserves further evaluation in a larger group of patients. Finally, a subset of patients with well differentiated, diploid tumor cells and negative axillary lymph nodes also showed a trend, although statistically not significant, of an improved prognosis when LEA.135 is expressed, again suggesting further study in a larger group of patients.

From the biologically functional point of view, the expression of LEA.135 on tumor cells of primary mammary carcinoma probably reflects an ability of these cells to perform the functions of differentiated cells. The result agrees with other reports which have described varying degrees of secretory activity, including lactational differentiation in mammary carcinomas. In conclusion, the expression of LEA.135 appears to be associated with relatively intact functional differentiation of neoplastic mammary epithelial cells, providing indirect evidence that the neoplastic process is not far advanced and that a more favorable prognosis may be anticipated.

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