A Monoclonal Antibody to a Human Breast Tumor Protein Released in Response to Estrogen

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ABSTRACT

A panel of monoclonal antibodies was produced against cell surface antigens of the MCF-7 human breast cancer cell line. The monoclonal antibodies were selected by their ability to bind to live, intact MCF-7 cells in solid-phase radioimmunoassay, and to bind to human breast cancer cells in paraffin sections. One monoclonal antibody, designated UCD/AB 6.11, identified two cellular antigens and one extracellular antigen from MCF-7, and bound to 18 of 20 breast cancers in paraffin sections. The two cellular antigens were associated with M, 54,000 and M, 56,000 proteins, which could be identified in Western blots, and were localized to the cell surface by immune precipitation of lactoperoxidase-iodinated plasma membrane proteins. The extracellular antigen, a M, 52,000 protein, was slightly more acidic and was only found in the media from estrogen-stimulated cells. Estrogen did not appear to have an effect on the production of the cell-associated antigens, M, 54,000 and 56,000 proteins. We postulate that the cellular antigens are precursors to the secreted M, 52,000 protein.

INTRODUCTION

Selection of breast cancer patients for endocrine therapy is, in part, based upon the presence of ER4 in their tumors (5, 6). However, the results of the ER assay are not totally predictive, since only 40 to 60% of patients with ER-positive tumors respond to estrogen therapy, and some 10% of patients with ER-negative tumors respond (13, 16). Clearly, other approaches are needed for evaluating the hormone responsiveness of breast cancers (14, 23).

The alternative approach of identifying estrogen-induced cell products has been pursued by several groups (1, 6, 7, 14, 23). In theory, the presence of an estrogen-sensitive product could be an indication that the cancer cell has fully functional ERs. Estrogen-sensitive polypeptides have been identified in the human breast cancer cell line MCF-7 (1, 14, 23). An estrogen-induced p24 has been identified in MCF-7 cells and a MCAb has been produced against it (1). Several polypeptides have been detected in the tissue culture fluid of the MCF-7 cell following estrogen stimulation (14, 24). However, no MCAb against the secreted polypeptides has been described. We developed a panel of MCAbs against MCF-7 cell surface antigens. One of the panel, UCD/AB 6.11, identified an antigen in the tissue culture fluid of estrogen-stimulated MCF-7 cells. The general characteristics of UCD/AB 6.11 and the antigens it detects are described here.

MATERIALS AND METHODS

Cell Lines

MCF-7 Cells. The MCF-7 human mammary tumor cell line was used to both immunize and screen for antibody production. This is a well-characterized cell line derived from a pleural effusion of a patient with metastatic breast cancer (21). It contains both estrogen (2) and progesterone receptors (9).

HBL-100. HBL-100 is a nonmalignant human breast epithelial cell line developed from a milk sample (20). It does not contain ERs (10).

186-NWT. 186-NWT is a human epithelial cell line developed in our laboratory from ascites fluid from a patient with metastatic breast cancer. It has not shown any breast cell markers and is ER negative.

P3x63Ag8.853. This mouse myeloma cell line is used as a hybridoma fusion partner for MCAb production. It does not produce immunoglobulin or immunoglobulin subunits (11).

Hybridoma Production

BALB/c mice were immunized by injection of 2 × 106 live MCF-7 cells once a week for 3 weeks. Three weeks after the third injection, the mice were boosted with another injection of live MCF-7 cells. Three days later, the spleen cells were harvested and fused with P3x63Ag8.853 myeloma cells using polyethylene glycol (19). The hybrid cells were hypoxanthine/aminopterin/thymine selected and were grown on a thymus cell feeder layer. Clones were established by end-point dilution. Cell lines were injected into pristane-primed BALB/c mice for ascites production.

Growth Media

Hybridoma cell lines were grown in Roswell Park Memorial Institute Tissue Culture Medium 1640 (Grand Island Biological Co.) Grand Island, NY) supplemented with 10% heat-inactivated horse serum, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 2 mM l-glutamine, and 25 μg gentamicin.

Human breast cell lines were maintained in Dulbecco’s modified Eagle’s minimal essential medium with either 5% calf or 5% horse serum, 1 μg insulin/ml, 25 μg gentamicin, and 100 μM 17β-estradiol (Sigma Chemical Co., St. Louis, MO).

Immunoperoxidase

Paraffin blocks of normal and malignant human breast tissue were obtained from the Department of Pathology, University of California Medical Center, Sacramento, CA. The antigens were detected in paraffin sections using the avidin:biotin complex method (17).

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HMFGMs

Delipidated HMFGMs were prepared by extracting the cream fraction of human milk with chloroform and ether (4).

Immunodiffusion

The MCAbs were isotyped, using the double-diffusion method of Ouchterlony. Supernatants from each clone and ascites fluid diluted 1:100 in PBS were reacted against antisera specific (Miles Laboratories, Elkhart, IN) for each immunoglobulin class: IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA.

Live Cell Solid-Phase RIA

Live MCF-7 cells were used in solid-phase RIA for first and second level screening (3). HBL-100 was used as a negative screen because it is nonmalignant and ER negative. HMFGM isolated from normal human milk was also used for a negative selection criterion. MCF-7 and HBL-100 cells were harvested from 75-cm tissue culture flasks by trypsinization, pelleted in 96-well tissue culture plates at 50,000 cells/well, and incubated for 18 to 24 hr at 37° in 5% CO2. Growth media were aspirated, 150 µl of growth medium containing 0.08% sodium azide were added, and cells were incubated for 30 min. Cells were rinsed with Hank's balanced salt solution containing 0.08% sodium azide and 5% cell serum (wash buffer); 100 µl of wash buffer were then added, and the cells were incubated 30 min. The cells were again rinsed with wash buffer and 50 µl of tissue culture fluid from a hybridoma culture, or diluted ascites was added and incubated for 1 hr. The cells were then rinsed twice with wash buffer and 50 µl (2 × 10^6 cpm) ^35S-rabbit anti-mouse IgG were added. After 1 hr, the cells were washed twice with wash buffer, and the positive wells were visualized by autoradiography.

Total Cell Protein Extraction

Cells grown to near confluency in 75-cm tissue flasks were washed twice with 10 ml PBS. The cells were then scraped from the flask with a rubber policeman in 10 ml PBS, and pelleted by centrifugation at 1500 rpm for 3 min. One ml of extraction buffer (0.10 M phosphate:0.5% Triton X-100:0.15 M NaCl:1 mM phenylmethylsulfonyl fluoride:0.02% NaN3) was added and incubated on ice for 15 min. The mixture was then centrifuged in a Beckman microfuge for 10 min, the supernatant was removed and stored at −20°, and an aliquot was removed for Bradford protein assay.

Protein Analysis

Proteins were analyzed by SDS-PAGE (12). Samples were applied in 50 µl of sample buffer [63 mM Tris (pH 6.8):10% glycerol:5% 2-mercaptoethanol:2.3% SDS] onto a 4% polyacrylamide stacking gel, and were electrophoresed for 4 hr into a 10 or 12% resolving gel with 0.2% SDS at a constant current of 200 mA/gel. The molecular weights of proteins were estimated by their mobilities relative to standard proteins of known molecular weight. Two-dimensional electrophoresis was as described by O'Farrell (18), except that SDS-PAGE in the second dimension was in a 10% resolving gel.

Immunoprecipitation

Formalin-fixed Staphylococcus aureus cells were pretreated to reduce protein sloughing from the cell surface by suspending the cells in an equal volume of PBS, pH 7.2, containing 10% (w/v) β-mercaptoethanol and 3% (w/v) SDS, and heated for 30 min at 95°. The cells were washed in SA buffer and resuspended in SA buffer to 10% (w/v).

Fifty µl of ^35S-methionine-labeled tissue culture fluid and 5 µl of 1:10 dilution of UCD/AB 6.11 ascites were mixed and incubated for 6 hr at 4°. Five µl of rabbit anti-mouse immunoglobulin were then added and incubated for 10 hr at 4°. One hundred µl of 10% treated S. aureus (above) were then added and incubated for 1 hr at 4°. The cells were then centrifuged in an Eppendorf microfuge for 5 min, washed twice with SA buffer, and 75 µl of SDS-PAGE sample buffer were added and incubated for 10 min at 90°. The mixture was then centrifuged in a Beckman microfuge, and 50 µl of supernatant were run in SDS-PAGE (4% running gel and 10% stacking gel). ^35S-Methionine-labeled protein bands were visualized by autoradiography.

Western Blots

A modification of the Western blot (22) was used in which the proteins were transferred from SDS-PAGE gels to nitrocellulose filters and identified by the MCA. After transfer to the nitrocellulose filters, excess protein-binding sites were blocked by soaking the filters in PBS containing 3% BSA. The antigen was located by incubating the sheet in 30 ml of PBS containing 1% BSA and 1 to 2 × 10^7 cpm of iodinated antibody for 1 hr. The filter was then rinsed, dried, and autoradiographed.

Analysis of Labeled Proteins

Cells were grown 10 or more days on 5% cell serum treated with dextran-coated charcoal to remove steroid hormones. Two days prior to testing, cells were trypsinized and plated at 4 × 10^6 cells/100-mm Petri dish. After 24 hr, various amounts of estradiol were added to the media to attain concentrations of 0, 1, 10, and 100 nM estradiol. The media were changed after 24 hr. After 48 hr of hormone treatment, the cells were washed twice with Hank's balanced salt solution. Serum-free medium, hormone supplemented as above, containing 10% of the normal methionine concentration plus 200 µCi of ^35S-methionine/ml were added. The cells and media were harvested after 6 to 12 hr of incubation.

Immunaffinity Chromatography

Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) was activated with cyanogen bromide, as described (15). Antibodies were purified from ascites by precipitation in 40% ammonium sulfate at 4° overnight. Tissue culture fluid was collected and made up to 2 ml in both EDTA and phenylmethylsulfonyl fluoride to inhibit proteolysis. Debris was removed by centrifugation at 5000 × g for 20 min, followed by filtration through glass wool and 0.22-µm filters. The sample was passed through a 1-ml column of Sepharose 4B before being passed through the affinity column at 20 to 30 ml/hr at 4°. Nonspecifically bound protein was eluted with 10 to 20 ml of 0.5 M urea in PBS. Antibiotin was eluted with 4-column volumes of 6 M guanidine·HCl, pH 1.5, neutralized, and dialyzed.

RESULTS

Production and Characterization of MCAbs. The hybridoma cell lines were initially selected by the reactivity of their supernatant fluid in the live cell solid-phase RIA. Cells in 87 of the 288 master wells produced antibodies which bound to live MCF-7 cells. Twenty-two of the 87 master wells did not react to HBL-100 cells or to HMFGM. Cells from 9 of the 22 wells were cloned by limiting dilution. Cells from several other wells producing antibodies against HBL-100 and MCF-7 cells were also cloned. For the purpose of this report, one MCAb, designated UCD/AB 6.11, is emphasized, and other MCAbs are used as quality controls or to illustrate specific data.

Fig. 1 illustrates the live cell solid-phase assay for clones UCD/ AB 6.11, 6.01, and 6.13. UCD/AB 6.11 and 6.01 titrated 1 × 10^-7 against live MCF-7 cells, but did not react with HBL-100 cells. In contrast, UCD/AB 6.13 bound to both MCF-7 and HBL-100 cells. UCD/AB 6.11 and UCD/AB 6.01 were isotyped as IgG1 (data not shown).

As seen on Coomassie stains, the SDS-PAGE protein patterns of the 3 breast cell lines were almost indistinguishable (Fig. 2a).
When the same proteins were transferred to nitrocellulose filters and reacted with UCD/AB 6.11 in Western blots, 2 antigens, p56 and p54, were detected in extracts of MCF-7 cells, but not in the other protein extracts (Fig. 2b). By way of contrast, UCD/AB 6.13 identified antigens in HBL-100, NWT-186, and MCF-7 cells, but not in HMFGM (Fig. 2c).

UCD/AB 6.11 also detected cytoplasmic antigens in paraffin sections of 18 of 20 human breast cancers, using the immunoperoxidase technique (Fig. 3a). Normal breast tissue adjacent to the cancer cells did not stain with UCD/AB 6.11. However, UCD/AB 6.11 did stain the epithelial cells in a normal prelactating breast (Fig. 3b). A more detailed description of the immunocytochemical results is in preparation.

Characterization of the Antigen. Since the MCAbs were initially selected using a live cell assay, the antigens were assumed to be on the cell surface. Further evidence supporting this assumption was obtained by immune precipitation of lactoperoxidase-labeled surface membranes (Fig. 4). UCD/AB 6.11 precipitated a radiolabeled protein, M, 54,000 antigen.

The 2 antigens identified by UCD/AB 6.11 in Western blots appeared to be in different concentrations (Fig. 2). A more accurate representation of the relative concentrations was obtained using several dilutions of MCF-7 proteins (Fig. 5). The relative intensity and relative mobility of the 2 antigens were dependent upon the protein concentrations. For example, at 150 µg of MCF-7 protein, p56 was not detected, but at 50 µg of protein, p56 was at the same apparent concentration as p54. At all other concentrations and by end-point titration, more radiolabeled UCD/AB 6.11 bound to p54 than to p56.

Separation of the MCF-7 proteins by 2-dimensional electrophoresis provided additional information. On Western blots of the 2-dimensional gels, UCD/AB 6.11 identified a series of at least 4 isoelectric forms of p56, and 7 forms of p54 (Fig. 6a). The p54 forms were slightly more acidic than the p56 forms.

MCF-7 Antigens in Tissue Culture Media. The tissue culture media from MCF-7 cells stimulated by estrogen were examined for antigens (Fig. 7). As in previous Western blots, the MCF-7 cell proteins contained antigens detected by UCD/AB 6.01 and 6.11. However, UCD/AB 6.11 detected high levels of p52 in the media, while UCD/AB 6.01 did not detect antigens in the tissue culture media.

The tissue culture antigen detected by UCD/AB 6.11 isoelectrically focused as a poorly resolved, closely packed group of perhaps 2 to 4 forms in 2-dimensional electrophoresis (Fig. 6b). When the MCF-7 whole-cell proteins were combined with tissue culture fluid in 2-dimensional Western blots, the relative size and charges could be evaluated (Fig. 6c). The antigen in the culture fluid was much more acidic and slightly smaller than the 2 cellular antigens. These size and charge characteristics were not as readily appreciated in the one-dimensional SDS-PAGE.

Since p52 was a major antigen released into the culture media by MCF-7 cells, it could be identified as metabolically labeled protein in the media (Figs. 8 and 9). In fact, several [35S]methionine-labeled peptides appeared in the cell supernatant (Fig. 8). However, radioimmune precipitation of the radiolabeled proteins with UCD/AB 6.11 greatly enriched for p52 (Fig. 8).

When labeled or unlabeled MCF-7 tissue culture fluids were passed through a Sephacryl 300 column, the p52 was largely concentrated in the void volume, implying that it is in a high-molecular-weight complex. Treatment of the complex with Triton X-100 reduced the antigenicity to an estimated M, 52,000 fraction (data not shown).

Effect of Estrogen Stimulation. When MCF-7 cells were grown in the absence of estrogen, metabolically labeled p52 was not found in the culture medium (Fig. 9). The level of antigen was also reduced below detection in Western blots (data not shown). When estrogen was added to unstimulated MCF-7 cells, p52 reappeared in the media (Fig. 9). When cells grown in the presence of estrogen were treated with the antiestrogen, nafoxidine, p52 disappeared from the media (Fig. 9).

In contrast, the MCF-7 cellular antigens p56 and p54 were not apparently affected by estrogen. Both antigens could be detected in MCF-7 cells grown in the presence or absence of estrogen (Figs. 4 and 5).

DISCUSSION

We report here the development of a monoclonal antibody, designated UCD/AB 6.11, that identifies a major immunoreactive protein which is released from estrogen-stimulated MCF-7 human breast cancer cells. Because of the potential clinical and scientific significance of an estrogen-sensitive breast cancer protein antigen, the subject is of intense interest (1, 7, 14, 23, 24). The protein described here, p52, could be related to either the estrogen-sensitive M, 52,000 glycoprotein reported by Westley and Rochefort (24), or to the low turnover estrogen-sensitive p50 reported by Mairesse et al. (14). Further work will be required to verify the relationships. However, the antigen described here is not related to the p24 described by Adams et al. (1).

The antigen identified by UCD/AB 6.11 is on a M, 52,000 methionine-containing protein released by MCF-7 cells. The immunoperoxidase studies indicate that the antigen is not restricted to MCF-7 cells, but is found in a majority of human breast cancers. Our preliminary results also indicate that the antigen is at higher concentrations in breast cancer than in normal quiescent breast, but that the antigen is not breast cancer specific.

From our data, the secreted p52 is antigenically related to 2 major cellular antigens, p56 and p54. The antigenic determinants appear to be on the cell surface, because UCD/AB 6.11 binds to the surface of live MCF-7 cells and immunoprecipitates surface-labeled antigens. The immunocytochemical observations suggest that the antigens also occur in the cytoplasm. Most significantly, these antigens appear in tumors with and without...
ERs. Furthermore, estrogen stimulation does not appear to affect the cytoplasmic levels of p56 and p54.

It is a reasonable working hypothesis that the cellular antigens identified by UCD/AB 6.11 are precursors to the secreted p52. Although the hypothesis must be verified by pulse chase experiments and a more detailed structural analysis, the multiple isoelectric forms and 2 molecular weights exhibited by the cellular antigens are consistent with a series of posttranslational modifications, leading to the secretion of a final more acidic, lower-molecular-weight polypeptide.

If this hypothesis is correct, the secretion, but not the production, of the p52 is controlled by estrogen. This implies that estrogen controls secretion at a posttranslational processing level. Perhaps, as described in the mouse mammary tumor virus structural polypeptides, estrogen controls the processing of p54 (8).

The demonstration that the secretion of p52 is controlled by estrogen raises the possibility that p52 might be used to determine whether a breast cancer has functional ERs. As an end product of estrogen action, qualitative or quantitative changes in p52 could be a useful marker for hormone-dependent tumors and may prove clinically useful. Since the antigen is secreted as a soluble complex, it may even prove useful as a serum marker for estrogen-dependent breast cancers.

REFERENCES


Fig. 2. One hundred µg of total cell proteins of MCF-7 cells (Lane 1), HBL-100 cells (Lane 2), NWT-186 cells (Lane 3), and a preparation of HMFGM (Lane 4) were electrophoresed into 12% SDS-PAGE. The gels were then stained with Coomassie blue (a), or electroeluted and exposed to radiolabeled MCAb UCD/AB 6.11 (b), or UCD/AB 6.13 (c). The distribution of radiolabeled MCAb was detected by autoradiography (b and c).

Fig. 3. Photomicrographs of breast cancer cells (a) and prelactating breast cells (b) stained using the immunoperoxidase technique for the detection of UCD/AB 6.11-related antigens. The tissues were fixed in B-5, embedded in paraffin, and counterstained with hematoxylin. Note that the reaction product in the tumor cells is diffuse while it is in discrete supranuclear granules in normal cells (arrows).
Fig. 4. Autoradiographs of lactoperoxidase-iodinated MCF-7 surface proteins separated in SDS-PAGE. Lanes 1 and 2 demonstrate total iodinated surface proteins, while lanes 3 and 4 contain proteins immune precipitated by UCD/AB 6.11, following a 2% Triton X-100 extraction. The MCF-7 cells were grown either in the presence of 100 nM estradiol (Lanes 1 and 3), or in the absence of estradiol (Lanes 2 and 4).

Fig. 5. Autoradiograph of a Western blot of SDS-PAGE, using iodinated UCD/AB 6.11 to detect antigens in whole-cell detergent extracts of MCF-7 cells grown in the presence (a) or absence (b) of estradiol. The extracts were diluted to protein concentrations of 150 μg (Lane 1); 100 μg (Lane 2); 75 μg (Lane 3); 50 μg (Lane 4); 25 μg (Lane 5); 10 μg (Lane 6); 5 μg (Lane 7).

Fig. 6. Autoradiographs of 2-dimensional Western blots using radiiodinated UCD/AB 6.11 to identify antigens in MCF-7 cells (a), the tissue culture fluid from MCF-7 cells (b) and both culture fluid and MCF-7 (c). The proteins were isoelectrically focused in the first dimension (IEF), and separated in SDS-PAGE in the second dimension (SDS). The MCF-7 cellular proteins were extracted from whole cells using Triton X-100. The tissue culture antigen was purified from a UCD/AB 6.11 immunoadfinity column. Blot c is a combination of proteins from Blots a and b.
Fig. 7. Autoradiographs (a and b) of Western blots. Coomassie blue-stained (c) total cell proteins (Lane 2), and secreted proteins (Lane 1) from MCF-7 cells grown for 24 hr on serum-free media containing 100 nm estradiol. The culture media were concentrated 10-fold by lyophilization, and 40 μl were applied to each lane and separated by SDS-PAGE. The MCF-7 cells were extracted with 2% Triton X-100, and 100 μg of protein were applied to each lane. Radiolabeled UCD/AB 6.01 was applied to Set a, and radiolabeled UCD/AB 6.11 was applied to Set b.

Fig. 8. Autoradiography of [3S]methionine-labeled proteins released into the culture medium by MCF-7 cells. Lane 1, total SDS-PAGE protein pattern; Lane 2, proteins immunoprecipitated by UCD/AB 6.11. Note that the electrophoresis running time into the SDS-PAGE was longer than in other gels in order to emphasize the p52 region (arrow).

Fig. 9. Autoradiography of SDS-PAGE of proteins in the culture fluid of MCF-7 cell grown with [35S]methionine. Arrow, p52. Lane 1, media from cells grown in the absence of estradiol; Lane 2, media from cells grown continuously without estrogen, and then treated with 100 nM estradiol 2 days prior to labeling; Lane 3, media from cells grown continuously with 100 nM estradiol; Lane 4, media from cells grown with estradiol and treated with 1.0 μM nafoxidine for 5 days. The nafoxidine was removed and the cells were treated with estradiol 2 days prior to labeling; Lane 5, media from cells grown with estrogen and treated with nafoxidine 7 days prior to labeling.
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