Cross-Reactivity of a Monoclonal Antibody Directed against an Estrogen-induced Protein in MCF-7 Human Breast Cancer Cells with Murine Leydig Cell Tumor Proteins

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ABSTRACT

An estrogen-responsive murine Leydig cell tumor (M5480A) was examined for the presence of cross-reactive proteins to a monoclonal antibody directed against a M, 24,000 estrogen-regulated protein in human breast cancer cells. Human breast tumor biopsies were used as controls for the cytosol preparations, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western blot conditions used in these experiments. The estrogen-regulated M, 24,000 protein was detected in sodium dodecyl sulfate-polyacrylamide gels of cytosols from four human breast cancer cell lines examined. Larger amounts of the M, 24,000 protein were present in the two estrogen-progesterone receptor-positive tumor biopsies in comparison to the two estrogen-progesterone receptor-negative samples. In addition, the two receptor-positive samples demonstrated an additional, less intense immunoreactive band at M, 21,000. Under identical conditions, the same monoclonal antibody bound to two major protein bands from sodium dodecyl sulfate-polyacrylamide gels of Leydig cell tumor cytosols at M, 56,000 and M, 86,000. Antibodies prepared from BALB/c mouse ascites fluid of animals bearing the parent myeloma cell line (P3X63NS1) exhibited no immunoreactivity against the human breast or Leydig cell tumor proteins. In light of the high degree of specificity which this monoclonal antibody exhibits, our results suggest that similar antigenic determinants may exist in these proteins from two distinct tumors.

INTRODUCTION

The successful treatment of breast cancer patients by endocrine therapy relies upon the identification of those tumors which are hormone responsive, with the presence of estrogen receptors being used as a criterion for hormone responsiveness. This, however, is not accurately predictive because only 50–60% of patients with estrogen receptor-positive tumors respond to estrogen therapy, while 6–10% of patients with estrogen receptor-negative tumors respond (1, 2). This discrepancy has led to the search for estrogen-induced proteins in human breast cancer as potential indicators of a functional estrogen receptor pathway. One such protein is the progesterone receptor which is estrogen regulated in cultured human breast cancer cells (3). Indeed the recently described estrogen-regulated proteins are also useful other than as potential markers of estrogen-responsive human breast cancer. For example, identification of the biochemical functions of these proteins will expand basic knowledge of the mechanisms by which this hormone elicits cellular responses. Estradiol has been shown to stimulate the synthesis and release of M, 66,000, M, 52,000, and M, 160,000 proteins in MCF-7 human breast cancer cells (5–7). Estradiol also stimulates the synthesis of a major M, 24,000 intracellular protein in these cells (8). Monoclonal antibodies have been generated against the M, 24,000 intracellular protein (9) as well as against the M, 52,000 secreted protein (5). These antibodies are of potential use not only as diagnostic and screening reagents but also as important probes into the mechanisms by which estrogens act.

Murine testicular Leydig cell tumors possess relatively high levels of estrogen receptor binding (10, 11) and are responsive to estradiol as evidenced by alterations in tumor growth (10) and in steroidogenesis (11). Although uncommon, a number of cases of human Leydig cell tumors have been reported in the literature (12). Recent evidence by Ciocca and Dufau (13) has demonstrated, using immunohistochemical techniques, a specific interaction of a monoclonal antibody directed against the M5480A cell M, 24,000 protein with human and rat Leydig cells. These investigators suggested that the antibody reacted with a M, 27,000 protein described previously.

The studies reported herein demonstrate a cross-reactivity between a monoclonal antibody to the MCF-7 human breast cancer M, 24,000 protein and two proteins (M, 56,000 and M, 86,000) in the estrogen-responsive M5480A murine Leydig cell tumor. Due to the reported specificity of the monoclonal antibody to M, 24,000 (14), similar antigenic determinants may exist among these proteins.

MATERIALS AND METHODS

Materials. The monoclonal antibody (G3.1), specific for the M, 24,000 protein of MCF-7 human breast cancer cells, was kindly provided by Drs. Dean P. Edwards and William L. McGuire of the Division of Oncology, Department of Medicine, University of Texas Health Science Center, San Antonio, TX under the auspices of NIH CA11378. Nitrocellulose membranes (0.2 μm) were obtained from Schleicher and Schuell (Keene, NH). Biotinylated antibody to mouse IgG and avidin D conjugated to horseradish peroxidase were purchased from Vector Laboratories (Burlingame, CA). Ascites control prepared from BALB/c mice given injections of pristane and P3X63NS1 myeloma cells was obtained commercially

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2 To whom requests for reprints should be addressed, at REPSCEND Labs (D-5), University of Miami School of Medicine, P. O. Box 016960, Miami, FL 33101.

The trivial names and abbreviations used are: estradiol, 1,3,5(10)-estratriene-3,17β-diol; sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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from Cappel Laboratories (West Chester, PA). The 4-chloro-1-naphthol was purchased from Aldrich Chemical Co., and the electrophoresis chemicals were from Bio-Rad Laboratories (Richmond, CA). Ultrapure urea was obtained from Schwarz-Mann (Orangeburg, NY). All other chemicals were of reagent grade and were obtained from Sigma Chemical Co. (St. Louis, MO).

**Tumor Preparations.** Murine Leydig cell tumors (M5480) were maintained by serial transplantation in C57BL/6J male mice at 14-day intervals (15). Human breast tumor samples were frozen at −70°C, pulverized, and stored at −70°C until preparation. Tumors were excised or thawed rapidly and diluted 1:4 with homogenization buffer containing protease inhibitors: 50 mm Tris-HCl, pH 7.4; 1 mm EDTA; 5 mm p-aminobenzamidine; 10 mm N-ethylmaleimide; and 1 mm phenylmethylsulfonyl fluoride.

The samples were subsequently homogenized by three 10-s bursts of a Brinkmann Polytron (setting 5) and centrifuged at 105,000 × g for 60 min. The cytosols were decanted and immediately prepared for electrophoresis.

**Polyacrylamide Gel Electrophoresis.** SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (16). Slab gels (1.5 × 140 × 160 mm) consisting of a running gel of 9.8% acrylamide; 10 mw N-ethylmaleimide; and 1 mw phenylmethylsulfonyl fluoride. Tumor Preparations. Murine Leydig cell tumors (M5480) were maintained by serial transplantation in C57BL/6J male mice at 14-day intervals (15). Human breast tumor samples were frozen at −70°C, pulverized, and stored at −70°C until preparation. Tumors were excised or thawed rapidly and diluted 1:4 with homogenization buffer containing protease inhibitors: 50 mm Tris-HCl, pH 7.4; 1 mm EDTA; 5 mm p-aminobenzamidine; 10 mm N-ethylmaleimide; and 1 mm phenylmethylsulfonyl fluoride. The samples were subsequently homogenized by three 10-s bursts of a Brinkmann Polytron (setting 5) and centrifuged at 105,000 × g for 60 min. The cytosols were decanted and immediately prepared for electrophoresis.

**Polyacrylamide Gel Electrophoresis.** SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (16). Slab gels (1.5 × 140 × 160 mm) consisting of a running gel of 9.8% acrylamide-0.26% bisacrylamide and a stacking gel of 4.9% acrylamide-0.13% bisacrylamide were polymerized with 0.08% ammonium persulfate. Electrophoresis was performed under conditions of constant current with stacking at 15 mA/gel and running at 30 mA/gel.

Following centrifugation, cytosol samples were diluted 1:1 with 2 × sample buffer to yield 62.5 mm Tris-HCl (pH 6.8), 2% SDS, 1 mm EDTA, 10% β-mercaptoethanol, 0.01% bromophenol blue, and 10% glycerol. In separate experiments, ultrapure urea was included to yield a final concentration of 7 mm. Next they were immediately immersed in a boiling water bath for 5 min, cooled to ambient temperature, and loaded directly on the gels without freeze-thawing. Each gel contained at least one lane with the following molecular weight standards: β-galactosidase (130,000); phosphorylase a (100,000); bovine serum albumin (68,000); ovalbumin (43,000); chymotrypsinogen (25,700); and myoglobin (17,200). Western Blots. Proteins were transferred from SDS-polyacrylamide gels to nitrocellulose membranes using a modification of the Western blot procedure of Burnette (17). Transfer was effected in 20 mm Tris base, 150 mm glycine, and 20% methanol at 6 V/cm for 16 h. Following transfer, the standard-containing nitrocellulose lanes were excised and stained in 0.1% Amido black 45, 45% methanol, and 10% acetic acid for 5 min. Rapid destaining was effected in 90% methanol-2% acetic acid for 2–5 min.

**For antibody reactions following transfer, nitrocellulose sheets** were incubated in 50 mm HEPES (pH 7.4), 5 mm EDTA, 150 mm NaCl, 0.05% Triton X-100, 0.25% gelatin, and 3% bovine serum albumin for 2 h at 37°C to block excess protein-binding sites. The sheets were then incubated with the anti-M, 24,000 protein antibody at a dilution of 1:10,000 for 24 h at 4°C in 50 mm HEPES (pH 7.4), 150 mm NaCl, 5 mm EDTA, 0.05% Triton X-100, 0.25% gelatin, and 1% bovine serum albumin. Following incubation, the sheets were washed 3 times in the same buffer followed by a 1-h incubation at 23°C with biotinylated horse antimouse IgG at a dilution of 1:2000. The sheets were washed 3 times and incubated with avidin-horseradish peroxidase at a dilution of 1:2000 for 30 min at 23°C. The sheets were then washed 4 times in 50 mm HEPES (pH 7.4)-150 mm NaCl followed by the addition of 0.06% 4-chloro-1-naphthol and 0.1% H2O2. Incubations with this substrate for 10-20 min were frozen in liquid nitrogen, pulverized, and homogenized in 10 volumes of solution 2 in a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at the lowest setting. Three 30-s bursts were required with 30 s cooling between bursts. The homogenate was centrifuged at 148,000 × g in a Sorvall OTD-65 ultracentrifuge (DuPont Instruments, Newtown, CT) for 30 min at 4°C to obtain the cytosol fraction.

Aliquots of cytosol (15 μl; 1–3 mg of protein per ml) were incubated in duplicate with increasing concentrations (0.05–5 nM) of either [2,4,6,7-3H]estradiol (specific activity, 90–100 Ci/mmol) or [17-α-methyl-3H]methyltrienolone (R-1881) (specific activity, 86 Ci/mmol), both from New England Nuclear, Boston, MA. Parallel samples were incubated in the presence of at least a 150-fold excess of either diethylstilbestrol or nonradioactive methyltrienolone. After 16–18 h of incubation at 4°C, bound and free ligand were separated by adding 0.4 ml of the following dextran-coated charcoal suspension: 0.25% charcoal (Sigma) and 0.0025% dextran clinical grade (Schwarz-Mann) in solution 1. The tubes were shaken vigorously in a reciprocating shaker at 4°C for 15 min. After centrifugation at 2000 × g for 10 min, 400-μl aliquots of the supernatant were counted in 10 ml of ACS (Amersham, Arlington Heights, IL) in a scintillation counter. The data obtained were plotted according to the method of Scatchard (18), and the results are given in fmol of receptor per mg of cytosol protein. Tumors were considered positive above 10 fmol/mg protein, negative below 3 fmol/mg protein.

**RESULTS**

As an internal control for the conditions of cytosol preparation, SDS-polyacrylamide gel electrophoresis, and Western blot analysis in these experiments, human breast tumor samples were included. As shown in Table 1, these samples had different levels of estrogen and progesterone receptor binding. Samples 1 and 2 had roughly equivalent levels of estrogen receptor binding, while sample 1 had more than a 2-fold higher level of progesterone receptor binding. Samples 3 and 4 had undetectable levels of estrogen and progesterone receptor binding.

Western blot analysis of these cytosols using the monoclonal anti-M, 24,000 protein antibody demonstrated the presence of the M, 24,000 protein in all four human breast tumor cytosols (Fig. 1). Equivalent amounts of protein were added to each sample well, but different levels of immunospecific staining were observed among the samples. The two receptor-negative samples (Fig. 1, C and D) stained less intensely for the M, 24,000 protein than did the receptor-positive samples (Fig. 1, Lanes A and B). The sample with the higher level of progesterone receptor binding (Fig. 1, sample 1, Lane A) stained more intensely than did the other receptor-positive sample (Fig. 1, sample 2, Lane B). In addition, a faint immunoreactive band at M, 21,000 was observed among the samples. The two receptor-negative samples (Fig. 1, C and D) stained less intensely for the M, 24,000 protein than did the receptor-positive samples (Fig. 1, Lanes A and B). As a control for the monoclonal antibody, antibodies purified from BALB/c mouse ascites fluid of mice bearing the parent myeloma cell line (P3X63NS1) were tested using Western blots of human breast tumor cytosol; no immunoreactivity was detected (Fig. 1, Lane E).

**Western blot analysis of the M5480A murine Leydig cell tumor**

**Table 1**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Estrogen binding* (fmol/mg protein)</th>
<th>Progesterone binding* (fmol/mg protein)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>379</td>
<td>333</td>
</tr>
<tr>
<td>2</td>
<td>333</td>
<td>140</td>
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<tr>
<td>3</td>
<td>&lt;3†</td>
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<tr>
<td>4</td>
<td>&lt;3†</td>
<td>&lt;3†</td>
</tr>
</tbody>
</table>

* These data represent the Bmax of the samples determined by Scatchard analysis of equilibrium binding data.
† No high affinity binding was detectable; tumors were considered positive above 10, borderline at 4–10, and negative below 3 fmol/mg protein.

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A, 100 ng; Lane B, 50 ng; Lane C, 20 ng; Lane D, 10 ng; Lane E, standards; Lane F, 100 ng of Leydig cell tumor cytosol In a control experiment as described in Fig. 1. Lane tumor cytosol; again no immunoreactivity was detected (Fig. 2).

Proteins at Mr, 86,000 and Mr, 56,000 were demonstrated two major cross-reactive bands. Proteins at Mr, 86,000 and Mr, 56,000 appeared to demonstrate some size heterogeneity upon increasing sample loading (Lanes A and B). The data shown are representative of four such experiments. Multiple individual Leydig cell tumors from three successive passages of the tumor yielded similar data. A control comparable to that used for the human breast tumor cytosol was performed using a murine Leydig cell tumor. These proteins do not appear to be aggregates of a smaller subunit because the inclusion of 7 M urea in the sample buffer had no effect on their relative electrophoretic mobilities. Although a recent report (13) demonstrated immunohistochemical localization in rat Leydig cells using a monoclonal antibody against the Mr, 24,000 protein and implied an interaction with a Mr, 27,000 protein, no biochemical data were presented. The studies reported herein raise some interesting possibilities regarding a possible relatedness between these proteins, which possess similar antigenic determinants. It will be of interest to determine if the Leydig cell tumor proteins are regulated by estrogens and if these proteins possess related sequences.

It is interesting that the intensity of immunoperoxidase staining and the presence of the Mr, 21,000 protein appeared to correlate with the presence of detectable estrogen-progesterone receptor binding. Clearly a large sample pool needs to be evaluated before such a correlation can be established. However, the presence of such a correlation would aid in the assessment of a functional estrogen receptor pathway and the selection of patients for endocrine therapy.

Estrogens have been shown previously to regulate the induction and growth (10) of murine Leydig cell tumors although the role of estrogens in normal Leydig cell function remains unclear. One group of investigators has hypothesized that estrogens modulate the response of Leydig cells to gonadotropins and specifically mediate the desensitization response of these cells to high levels of gonadotropins (19, 20). However, this hypothesis is not supported by results from a number of laboratories (21–26). Preliminary data by Melner and Puett (27) have demonstrated several major estrogen-induced proteins (Mr, 135,000, Mr, 50,000, and Mr, 36,000) in murine Leydig cell tumors. Sato et al. (28) have also demonstrated estrogen-induced secretory proteins (Mr, 36,000 and Mr, 34,000) in an estrogen-responsive BALB/c mouse Leydig cell line (T 124958-R). The identification, purification, and characterization of estrogen-regulated proteins in these cells should yield insight into the role of estrogens in Leydig cell function as well as the mechanisms by which these steroids induce tumor formation. The results presented herein identify proteins of interest in Leydig tumor cells for subsequent purification and characterization studies. The monoclonal antibodies will be important aids to these subsequent studies.

Fig. 1. Western blot transfers of human breast tumor cytosol samples following SDS-polyacrylamide gel electrophoresis in 10% gels. Reactivity with anti-Mr, 24,000 monoclonal antibody was detected with anti-mouse IgG-biotin followed by avidin-horseradish peroxidase and 4-chloro-1-naphthol. Each sample contained 100 µg of protein. Lane A, sample 1; Lane B, sample 2; Lane C, sample 3; Lane D, sample 4; Lane E, sample 5 in a control experiment in which the cytosol has been reacted with antibodies purified from BALB/c mouse ascites fluid of mice given injections of the unfused, parent myeloma cell line (P3X63NS1).

Fig. 2. Western blot transfer of M5480A murine Leydig cell tumor cytosol samples following SDS-polyacrylamide gel electrophoresis in 10% gels. Reactivity with anti-Mr, 24,000 monoclonal antibody was detected using a murine Leydig cell line. The data shown are representative of four such experiments. Multiple individual Leydig cell tumors from three successive passages of the tumor yielded similar data. A control comparable to that used for the human breast tumor cytosol was performed using a murine Leydig cell tumor cytosol; again no immunoreactivity was detected (Fig. 2, Lane F).

The Leydig tumor cell Mr, 86,000 and Mr, 56,000 proteins demonstrated relative mobilities identical to those shown in Fig. 2 in SDS-polyacrylamide gel electrophoresis following treatment of the samples in sample buffer containing 7 M urea (final concentration).

REFERENCES

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