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 tumor biopsies 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 addition of this selection criterion has raised the response rate of endocrine therapy to 80% (1, 4). In addition, a number of other estrogen-regulated proteins have been described. 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, 46,000, 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 MCF-7 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.

3 The trivial names and abbreviations used are: estradiol, 1,3,5(10)-estratriene-3,17β-diol; sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazinenucleic acid.
from Cappel Laboratories (West Chester, PA). The 4-chloro-1-naphthol was purchased from Aldrich 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 x 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 (15 x 140 x 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 x 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, urea gel was included to yield a final concentration of 7 M. Next they were immediately immersed in a boiling water bath for 5 min. After 3 min in boiling water, the gels were 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 x g for 60 min. The cytosols were decanted and immediately prepared for electrophoresis.

**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 Mr 24,000 protein than 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 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>
</thead>
<tbody>
<tr>
<td>1</td>
<td>379</td>
<td>333</td>
</tr>
<tr>
<td>2</td>
<td>335</td>
<td>140</td>
</tr>
<tr>
<td>3</td>
<td>&lt;2</td>
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<tr>
<td>4</td>
<td>&lt;2</td>
<td>&lt;2</td>
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</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.
cytosols using the anti-\( M \), 24,000 antibody revealed two major cross-reactive bands. Proteins at \( M \), 86,000 and \( M \), 56,000 were detected using this antibody (Fig. 2). The \( M \), 56,000 protein appeared to demonstrate some size heterogeneity upon increasing sample loading (Lanes A and B). The data shown are representative of three 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 E).

DISCUSSION

These results demonstrate a cross-reactivity between a monoclonal antibody, which was directed against an intracellular human breast tumor \( M \), 24,000 protein and two intracellular proteins (\( M \), 86,000 and \( M \), 56,000) from the M5480A 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 \( M \), 24,000 protein and implied an interaction with a \( M \), 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 \( M \), 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 Meiner and Puett (27) have demonstrated several major estrogen-induced proteins (\( M \), 135,000, \( M \), 50,000, and \( M \), 36,000) in murine Leydig cell tumors. Sato et al. (28) have also demonstrated estrogen-induced secretory proteins (\( M \), 36,000 and \( M \), 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.

REFERENCES


LEYDIG CELL TUMOR PROTEINS


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