Monoclonal Antibody to Human Carcinoma-associated Protein Complex: Quantitation in Normal and Tumor Tissue

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ABSTRACT

We have identified and quantitated a tumor protein complex, TSP-180, on murine carcinomas with two monoclonal antibodies (MoAbs) (Cancer Res., 46: 707-712, 1986). One of the two MoAbs, 135-13C, recognizes a TSP-180-like protein complex on several human carcinomas in culture. MoAb 135-13C has been used to purify the human TSP-180 complex from A431 cells and the purified material used to immunize F344 rats to produce another MoAb, 439-9B, to the human TSP-180 complex. This MoAb does not precipitate the murine TSP-180 or bind to murine cells. Both MoAbs 135-13C and 439-9B precipitated the same proteins from A431 cells but did not compete with each other for binding sites, indicating that they recognize different epitopes on the same protein. The two MoAbs have been used in a two-site assay to quantitate TSP-180 proteins on human cells and tissues. Carcinoma cell lines A431, SW948, and A549 all give high values (46 to 443 ng/mg of protein) while murine tumors, a human melanoma, and human fibroblasts are negative (≤10 ng/mg of protein). Most tissues from autopsy of 2 normal individuals are negative for human TSP-180 at the levels tested (≤10 ng/mg of protein). Some organs have intermediate range expression: spleen, 5 to 111 ng/mg of protein; colon, 24 to 111; and small intestine, 39 to 99. One primary colon and one larynx tumor were positive (144 to 372 ng/mg of protein) while 5 breast carcinomas, a stomach tumor, a metastatic melanoma, and a kidney tumor were negative. These data indicate that human TSP-180 is a tumor associated antigen preferentially expressed in certain malignant carcinomas of diverse origin. The potential for TSP-180 as a tumor marker requires further study.

INTRODUCTION

Early stages of tumor progression are characterized by genotypic and phenotypic instability (1, 2). The phenotypic variations include quantitative alterations like the overexpression of specific proteins (3). Recently we demonstrated that the expression of a tumor antigen, in clones derived from Lewis lung carcinoma, is correlated with the metastatic potential of this tumor (4). This tumor antigen defined by MoAb5 135-13C (5) is expressed on a complex of glycoproteins ranging in molecular weight from 116,000–204,000. This group of related molecules is named the TSP-180 complex. TSP-180 proteins are expressed on the surface of several lung carcinoma and B16 melanoma cell lines in mice. Quantitation of TSP-180 from primary lung tumors using a two-site MoAb assay indicated a correlation between malignant potential and amounts of TSP-180 proteins expressed (4, 6). Recently we demonstrated that a human epidermoid carcinoma cell line (A431) also expresses a TSP-180-like protein complex. The complex from human cells reacts with one MoAb (135-13C) but fails to react with the other MoAb (346-11A) to the mouse TSP-180 complex. Since one MoAb, 135-13C, cannot be used to quantitate the expression of these proteins in normal human and tumor tissues, we developed a new MoAb to a second epitope on the human TSP-180 complex of proteins. In this paper we report that the TSP-180 complex from human cells is found in small amounts in normal tissues (colon, spleen, small intestine) and in larger amounts in some primary carcinomas (colon, larynx) and tumor lines (SW984 and A549).

MATERIALS AND METHODS

Cell Lines and Tissues. A BALB/c spontaneous lung carcinoma (line 1) (7) and human tumor cell lines, epidermoid carcinoma (A431) (8), lung carcinoma (A549) (8) obtained from Dr. W. Yang, Biology Division, Oak Ridge National Laboratory, and melanoma (WM 164) (9) obtained from Dr. L. Washburn, Oak Ridge Associated Universities, were grown in McCoy's Medium 5A with 10% fetal bovine serum. The colon carcinoma cell line (SW948) (10) obtained from Dr. L. Washburn was grown in L-15 medium with 10% fetal bovine serum. The breast tumor cell line (MCF-7) (11) from Dr. J. K. Selkirk, Biology Division, Oak Ridge National Laboratory, was cultured in Eagle's minimal essential medium with Hanks' balanced salt solution with 10% fetal calf serum. Leukemia cell line (K562) from Dr. C. B. Lozzio, University of Tennessee, was grown in Dulbecco's modified Eagle's minimal essential medium with 20% fetal bovine serum (12).

SP2/0 myeloma (13) and 135-13C hybridoma were grown in Dulbecco's modified Eagle's minimal essential medium with 20% fetal bovine serum. The human fibroblast line (HSBP) (14) was obtained from Dr. J. D. Regan, Biology Division, Oak Ridge National Laboratory, and was grown in McCoy's medium with 10% fetal bovine serum. Normal human and tumor tissues were obtained from autopsies or surgical operations and immediately frozen. The tissues were thawed at 4°C in PBS containing NP-40 (0.5%), PMSF (10 μg/ml), and NaN3 (1 mM) and homogenized with a Polytron homogenizer. The homogenates were cleared in a Sorvall centrifuge (10,000 rpm for 10 min at 4°C) and the supernatant was transferred to tubes and stored at −80°C prior to analyses.

Purification of TSP-180. The MoAb 135-13C (5) was purified from ascites fluid by precipitation with 50% saturated ammonium sulfate and chromatography on DEAE-cellulose (15) with further purification on high-performance liquid chromatography. The purified MoAb 135-13C (5 mg of MoAb per ml of beads) was coupled to epichlorhydrin-cross-linked Sepharose 4B beads after activation with cyanogen bromide (16). Excess MoAb was removed by washing the beads with 3 M KSCN containing 0.5% NP-40 and then 3 times with 0.5% NP-40 in PBS before use. TSP-180 was purified from A431 cell lysate (10% w/v) in PBS with NP-40 (0.5%), PMSF (10 μg/ml), NaN3 (1 mM), DNase II (10 μg/ml), MgCl2 (2 mM), and sonication.

Clarity of the cell lysate was performed by incubation with Sepharose beads coupled to MoAb 135-14 (MoAb nonspecific for human cells) for 2 h at 4°C with gentle mixing (10 ml of A431 cell lysate with 0.1 ml of Sepharose beads). After centrifugation at 800 × g for 2 min the supernatant was transferred to Sepharose beads coupled to MoAb 135-13C and TSP-180 was recovered as described previously (6).

Immunizations and Hybridoma Screening. Fischer 344 rats were

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3) The abbreviations used are: MoAb, monoclonal antibody; TSP-180, tumor surface protein, M, 180,000; PBS, 0.1 M sodium phosphate, pH 7.6, and 0.15
M NaCl; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; BSA, bovine serum albumin; TAA, tumor associated antigen; CEA, carcinoembryonic antigen; NP-40, Nonidet P-40.

S. J. Kennel et al., personal communication.
immunoprecipitation. The labeled lysate was divided into three parts and each was incubated at 4°C for 2 h with Sepharose beads (50 μl) containing 5 mg/ml purified MoAb 135-13C, 439-9B, or 135-14. The supernates were recovered and retreated with fresh beads to ensure immunodepletion of the antigens. Depleted supernates were then recovered and analyzed by immunoprecipitation and SDS-PAGE as described above.

Two-Site Assay. MoAb 135-13C was diluted in 50 mM NaHCO3 (pH 9.6) and incubated (1 μg/well) in Immunolon 2 Removawell strips (Dynatech Laboratories) overnight at 4°C with gentle mixing. Then 500 μg of BSA diluted in PBS were added (100 μl/well) for 1 h at 4°C with gentle mixing to saturate binding sites. Unbound material was aspirated from the wells and 50 μl of serial dilutions of homogenates from cell lines and tissues to be analyzed were added for 2 h at 4°C. The homogenates were diluted in PBS with BSA (5 mg/ml) and NP-40 (0.05%). The wells were washed in PBS and 50 ng of 125I-MoAb 439-9B diluted in PBS with BSA (5 mg/ml), NP-40 (0.05%), and 1% normal rabbit serum were added to each well for 2 h at 4°C. Then the wells were washed in PBS, transferred to 12 × 75 mm glass tubes, and counted in a gamma counter. All of the results of binding were calculated as the average of duplicate samples.

RESULTS

Purification of TSP-180 Proteins from A431 Cell Lines. Purified MoAb 135-13C coupled to Sepharose 4B was used to purify TSP-180-like proteins from a crude lysate of A431 cells grown in vitro. The proteins were recovered by elution of immunoaffinity-absorbed proteins with 3 M KSCN and 0.5% NP-40. Experience with murine TSP-180 indicates that the epitope for 135-13C is destroyed by treatment with 3 M KSCN, thus giving excellent yields of release from the immunosorbent (6). Approximately 50 μg of purified proteins were recovered from a lysate of 5 × 10⁶ A431 cells. The purified products were analyzed on 4–10% acrylamide gradient SDS-PAGE in reducing conditions. The results show that at least four bands of the TSP-180 protein complex are present (Fig. 1, slot 1).

Production and Characterization of MoAb. TSP-180 proteins were used to immunize F344 rats. The spleen cells from the rats were fused with SP2/0 myeloma cells and hybridomas were cultured and screened for antibody production by indirect binding. The supernatants from each hybridoma were tested on A431 cell lines and human fibroblasts were grown in 96-well plates. Only one hybridoma culture, 439-9, was identified as producing antibody that reacted against A431 cells but not control fibroblast cells. This culture was cloned and named 439-9B. After purification from ascites fluid, the class and subclass of MoAb 439-9B (IgG 2b) were determined by immunodiffusion.

Immunoprecipitation for the Characterization of Antigens. In order to determine the proteins recognized by this MoAb, A431 cells were surface radiolabeled using lactoperoxidase, solubilized with NP-40, and subjected to immunoprecipitation with different MoAbs. The analysis of immunoprecipitates in SDS-PAGE followed by autoradiography (Fig. 1) indicate that two MoAbs, 439-9B (slot 3) and 135-13C (slot 2), both recognize TSP-180 proteins. These proteins are not immunoprecipitated by MoAb 135-14 (slot 4) (a MoAb with no known binding specificity) or goat anti-rat IgG secondary antibody (slot 5), used as negative controls. Four bands at molecular weights of 214,000, 188,000, 148,000, and 128,000 are recognized by both MoAbs. These labeled bands comigrate with bands purified by immunoaffinity chromatography (slot 1). The same banding pattern is obtained when different detergents are used for solubilization or when samples are “aged” to amplify proteolysis. Prolonged proteolysis destroys all of the bands with no obvious

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binding, A431 cells have about $2.7 \times 10^5$ MoAb binding sites expressed on the surface of viable cells. To demonstrate that 135-13C and 439-9B MoAbs recognize different epitopes on the same molecules, reciprocal competition binding assays were performed (Fig. 3). In the first experiment A431 cells cultured in 6-well plates were treated with different dilutions of ascites fluid containing 135-13C or 439-9B antibodies and the $^{125}$I-MoAb 135-13C was added to each well. The result shows that ascites fluid 135-13C competes for binding of $^{125}$I-MoAb 135-13C but that ascites fluid 439-9B does not compete (Fig. 3B). In addition, the reciprocal experiment shows that ascites fluid 135-13C does not compete for the binding with $^{125}$I-MoAb 439-9B MoAb whereas 439-9B ascites fluid yields complete competition (Fig. 3A).

Banding patterns in SDS-PAGE of immunoprecipitates indicate that both antibodies recognize the same proteins (Fig. 1, slots 2 and 3). In order to demonstrate this directly, an immunodepletion experiment was performed. $^{125}$I-labeled A431 proteins were depleted of proteins reacting with each MoAb by incubation of the labeled proteins with Sepharose beads coupled with each purified MoAb and a control. The resultant depleted supernatants were then tested for immunoprecipitation with each MoAb. SDS-PAGE analysis of these immunoprecipitates is presented in Fig. 4. A431 proteins depleted using 439-9B beads no longer react with either of the MoAbs (slots 1 and 2). Similarly, A431 proteins depleted using 135-13C beads have little reaction with either MoAb (slots 4 and 5). A431 proteins incubated with control 135-14 beads show reactivity of all A431 TSP-180 bands with each MoAb. These results demonstrate that 135-13C and 439-9B MoAbs bind to different epitopes on TSP-180 proteins.

**MoAb Characterization.** In order to demonstrate that MoAb 439-9B was specific for human cells, direct binding assays with $^{125}$I-MoAb 439-9B were performed on A431, line 1, and human fibroblast (HSBP) cell lines (Fig. 2). The results show that MoAb 439-9B has a high affinity for human carcinoma cell line A431 but does not bind to line 1 cells or human fibroblasts. Antigen turnover at 37°C is negligible since similar results are obtained when incubations are carried out at 4°C. Assuming a molecular weight of 160,000 for MoAb 439-9B and a plateau level of binding of 5 ng/well, we calculate that at saturation accumulation of one at the expense of the other.6

6 S. J. Kennel, manuscript submitted.
7 Unpublished data.
Fig. 4. SDS-PAGE (4–10% gradient) analyses of immunodepletion/immunoprecipitation of "I-A431 proteins. Labeled proteins were depleted by Sepharose beads coupled to MoAb 439-9B (slots 1–3), MoAb 135-13C (slots 4–6), or control MoAb 135-14 (slots 7–9). The resultant depleted supernates were subjected to indirect immunoprecipitation with MoAb 439-9B (slots 1, 4, and 7), MoAb 135-13C (slots 2, 5, and 8), or control MoAb 135-14 (slots 3, 6, and 9).

Fig. 5. Two-site MoAb assay for the TSP-180 complex in human tumors and cell lines using MoAb 135-13C attached to Immulon strips and "I-MoAb 439-9B as a detection antibody. Lysates analyzed: A431 (O), colon tumor 1982 (A), larynx (O), A549 (D), breast tumor (•), or HSBP cells (A). Bars, range of duplicate determinations.

Quantitation of Human TSP-180 Proteins on Cell Lines and Human Tissue. Quantitation of TSP-180 proteins in normal human and tumor tissues was performed with a two-site assay. Purified MoAb 135-13C bound to Immulon 2 Removawell strips was used to concentrate TSP-180 proteins from cell and tissue lysates and "I-MoAb 439-9B was used to detect the bound antigen. Conditions were tested to determine which antibody should be used on the solid support as a concentration antibody and which should be used as a detection antibody to give the greatest sensitivity. We also tested different concentrations of detection antibody. Data from a standard assay using optimal conditions are shown in Fig. 5. A431 cells give a positive signal with near linear response between 2 and 10 ng of "I-439-9B antibody bound while HSBP cells give background levels. Data in Fig. 5 also show the result of the two-site assay performed on some of the tumor cells and primary tumors. Lysates from the A431 cell line and primary colon and larynx tumors show high levels (>100 ng/mg of protein) of binding of "I-MoAb 439-9B. No binding is detected with the lysate of the HSBP cell line (negative control). These values correspond to <5000 molecules/cells. These results are reported also in Table 1 (cell lines) and Table 2 (tumor tissue) where it is possible to compare the distribution of TSP-180 proteins in all tumors we tested. Each curve is converted to a single number (ng "I-MoAb bound/mg lysozyme protein added). Values are calculated at the point where 2 ng of "I-MoAb 439-9B are bound, the beginning of the linear portion of the curve. This value was found to be reproducible in replicate samples done in the same assay (data not shown). Variation as much as 2-fold occurs on replicate samples assayed on different days. A standard curve was included in different assays to control for the effects of this day-to-day variation when data from several assays are to be compared.

Most normal tissues were essentially negative (<10 ng/mg of protein) or showed low levels of binding (Table 3). Normal spleens from both autopsy samples were positive and one (the male) of two normal colon samples was positive. Very low levels (~20 ng/mg of protein) were also detected in esophagus, gall bladder, intestines, prostate, and breast tissues. Several additional samples of tissues giving positive results were obtained and tested (Table 4). These average values are similar to those determined in the preliminary tests (Table 3). The relatively large range from individual to individual is not an artifact of the test or sampling since in many cases 2 or 3 replicate tests were done on separate samples of a single specimen. Values for each of these replicate tests vary less than 20%.

Table 1 TSP-180 concentration in human tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>TSP-180 (ng/mg protein)*</th>
</tr>
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<tbody>
<tr>
<td>A431</td>
<td>443</td>
</tr>
<tr>
<td>SW948</td>
<td>312</td>
</tr>
<tr>
<td>A549</td>
<td>46</td>
</tr>
<tr>
<td>MCF7</td>
<td>18</td>
</tr>
<tr>
<td>WM164</td>
<td>&lt;10</td>
</tr>
<tr>
<td>K562</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* TSP-180 is quantitated as the amount (ng) of "I-MoAb 439-9B bound in the two-site assay per mg of lysate protein analyzed.

Table 2 TSP-180 in human primary tumors

<table>
<thead>
<tr>
<th>Primary tumor</th>
<th>TSP-180 (ng/mg protein)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon 1982</td>
<td>372</td>
</tr>
<tr>
<td>Colon 2112</td>
<td>29</td>
</tr>
<tr>
<td>Larynx</td>
<td>144</td>
</tr>
<tr>
<td>Endometrium</td>
<td>21</td>
</tr>
<tr>
<td>Pelvic mass</td>
<td>16</td>
</tr>
<tr>
<td>Stomach</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Metastatic melanoma</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Kidney</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Breast 46</td>
<td>&lt;10</td>
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<td>Breast 4353</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Breast 4358</td>
<td>&lt;10</td>
</tr>
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</table>

* See Table 1 for method of calculation.

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DISCUSSION

A number of human TAAs have been identified by reaction with monoclonal and polyclonal antibodies. Only a few quantitative assays have been developed (22–24). Since many, if not all, TAAs are also expressed on normal cells, a quantitative assay is essential for understanding of altered expression of these proteins. TSP-180 is a protein complex identified on the surface of murine lung tumor cells (5). Its concentration in cell lines and primary lung tumors is correlated with malignant potential (4, 6). One of two different MoAbs to TSP-180 in mice recognizes TSP-180-like proteins on several human carcinoma cell lines. The protein complexes from murine and human sources are related in that they show a common epitope defined by MoAb 135-13C, that they have 4–5 protein bands of each complex is phosphorylated. They differ in the individual molecular weights of each band and in reactivity with two other MoAbs. MoAb 439-9B binds only to human TSP-180 while MoAb 346-11A binds only to mouse TSP-180. It is clear, however, that most normal tissues give low or negative results. The large range of values among particular normal tissues from different individuals is unexpected. The range for spleen and colon may be due to undiagnosed conditions with these individuals. More samples from normal individuals need to be tested to confirm these results.

Some, but not all, of the primary tumors tested are positive. It is of interest that only 1 of 2 colon tumors are positive. One possible explanation is that different types of this tumor class can be distinguished by this assay. Alternatively, some normal adipose tissue may have been included in one of the samples which would dilute the amount of TSP-180 detected when normalized to total protein. Many more samples will need to be analyzed to demonstrate a correlation between tumor type and TSP-180 expression.

Quantitation of CEA (25–27), CA19-9 (28), and the transferrin receptor (29, 30) have proven useful in several aspects of diagnosis and monitoring of treatment of disease. TSP-180 is different from these tumor markers, CEA, epidermal growth factor receptor, transferrin receptor, or CA19-9 in molecular structure. Furthermore, TSP-180 is not found in soluble form like CEA or CA19-9 or on fibroblast type cells like the epidermal growth factor receptor or transferrin receptor. Thus, the fact that TSP-180 is different in molecular structure from any of the known TAAs and its apparently preferential expression on certain carcinomas make it unique. The assay for TSP-180 may serve some specialized purpose for tumor categorization or assessment of malignant potential when more data are available. Studies on the usefulness of TSP-180 as a diagnostic marker and on the basic question of gene regulation and molecular function are in progress.

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