Identification and Characterization of a Rat Hepatic Oncofetal Membrane Glycoprotein

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ABSTRACT

A major interest of our laboratory is to delineate the pathways leading to experimentally induced liver cancer in the rat. Although the cellular progenitors of primary hepatocellular carcinoma remain controversial, current findings suggest that proliferation of chemically initiated liver epithelial cells gives rise to hepatic nodules, a rare population of which eventually progress to carcinoma. Presently, the availability of cell surface markers that are closely associated with malignant progression is needed for the identification, isolation, and further characterization of these rare malignant cells. In this paper, we describe two new monoclonal antibodies (MAbs), MAb 324.5 and MAb 324.9, that recognize a novel oncofetal membrane glycoprotein, designated TuAg1. MAbs 324.5 and 324.9 were produced using three different transplantable hepatocellular carcinoma cell lines during immunization and screening. MAbs 324.5 and MAb 324.9 were shown to be reactive with different epitopes on TuAg1 by competitive immunoprecipitation assays combined with results from immunodepletion analysis and one-dimensional V-8 peptide maps. TuAg1 showed variations in molecular weight from 78,000 to 92,000, and a marked heterogeneity in pl, with charge variants ranging between 4.3 and 6.0. The 324.5-epitope was not expressed at detectable levels in any adult normal tissues or during liver regeneration but was transiently expressed during fetal liver development as shown by indirect immunofluorescence analysis of frozen tissue sections. In contrast, the 324.9-epitope was observed on nerve fibers and ganglia and on sperm tails in the adult rat and also appeared independently of the 324.5-epitope during fetal development. Although normal hepatocytes did not express TuAg1, isolated hepatocytes became positive during the first 24 h of primary culture. Attempts to modulate the in vitro expression of TuAg1 were unsuccessful; however, TuAg1 was lost within 7 days following ectopic transplantation of cultured hepatocytes into the pancreas. During the carcinogenic process, TuAg1 was expressed by a rare population of hepatic nodules, by many primary liver tumors, and by all lung metastases examined. Taken together, these observations suggest that the in vivo constitutive expression of this novel oncofetal membrane antigen is closely associated with acquisition of the malignant phenotype during hepatocarcinogenesis.

INTRODUCTION

In recent years a number of model systems have been developed in the rat to study the cellular pathways leading to PHC3 (for review, see Refs. 1 and 2). Using these models it has been shown that, following exposure to a carcinogen, chemically altered liver epithelial cells proliferate to form foci and nodules in the presence of a tumor promoter (3). However, when the promoting stimulus is removed, many of the preneoplastic lesions remodel until they morphologically and phenotypically resemble the surrounding parenchyma (4), while a rare population of nodules appear to progress, perhaps following additional cellular alterations, to carcinoma (2). In order to elucidate lineage relationships during the neoplastic process, our laboratory and others have employed monoclonal antibody technology to identify a number of markers expressed by different cell populations during hepatocarcinogenesis (5–9). Using this approach we previously established an antigenic relationship between a subpopulation of hepatic foci, nodules, and tumors and oval cells, a preneoplastic cell population displaying characteristics of hepatic stem cells (10). These findings, taken together with the recent results described by Braun et al. (11) and Evarts et al. (12–14), have suggested that, in addition to chemically altered hepatocytes, oval cells are also one of the cellular progenitors of liver cancer in some model systems. Regardless of the cellular precursor of PHC, the identity of the rare hepatic nodules from which tumors eventually arise remains unknown. The availability of cell surface markers that are closely associated with malignant progression is needed to identify and isolate these rare neoplastic cells for further characterization.

In this paper, we described the characterization of a novel tumor-associated antigen, designated TuAg1. TuAg1 is a membrane glycoprotein bearing two cell surface epitopes defined by MAbs 324.5 and 324.9. Both epitopes are expressed by a rare subpopulation of HN, by many PHC, and by all lung metastases and THC as well as by hepatocytes in primary culture but not by intact adult liver, very early preneoplastic hepatic lesions, or any other adult rat tissues. The 324.9-epitope is expressed independently at high levels on sperm tails and on nerve fibers and ganglia in many normal tissues. TuAg1 is also transiently expressed during fetal development and thus has the characteristics of an oncofetal antigen.

MATERIALS AND METHODS

Isolation of Tissues and Cells. Normal adult tissues were obtained from male ACI rats (Harlan Sprague Dawley, Indianapolis, IN) fed ad libitum a basal 101 purified diet (Dyet's, Inc., Bethlehem, PA). Tissues were excised, cut into 2- to 5-mm blocks, snap frozen in hexane, chilled by a dry ice-acetone bath and stored at −80°C. Fetal liver tissue was isolated at time zero of Days 12.5 and 13 to 20 of gestation and snap frozen in O.C.T. embedding compound. Female rats were paired with males for 2 to 4 h in the morning, after which time vaginal smears were immediately performed. The presence of sperm in the vagina was taken as time zero of Day 0 of gestation. Regenerating liver was isolated from adult male F-344 rats (Charles River, Wilmington, DE) at 3.0-h intervals (3 to 72 h) following a two-thirds partial hepatectomy performed as described by Higgins and Anderson (15).

Normal ACI hepatocytes were isolated by collagenase dissociation as previously described (5). Primary cultures of hepatocytes were maintained on plastic dishes coated with rat tail collagen (16). The basal medium was CEM 2000 (Scott Laboratories, West Warwick, RI) supplemented with 10% FBS (Armour Pharmaceuticals, Tarrytown, NY) and 50 μg/ml of Gentamycin (GIBCO Laboratories, Grand Island, NY). Other additions to the medium or substratum conditions are...
described in Table 1. In some experiments, freshly isolated hepatocytes or cultured hepatocytes (24 h) were also ectopically transplanted into the pancreas of male ACI rats (see below). For these experiments, cultured hepatocytes were removed from dishes by a 5- to 10-min digestion at 37°C with 0.05% collagenase in PBS.

Pancreatic acinar cells were isolated by collagenase digestion using the method of Oliver (17). Acinar cells were plated on plastic slides coated with Matrigel basement membrane (Collaborative Research, Inc., Bedford, MA) diluted 1:1 with PBS. Because of the difficulties of obtaining accurate cell counts because of aggregation, acinar cells were plated at several dilutions and allowed to attach for 2 h, and confluent monolayers were chosen for subsequent analysis. Cultures were maintained in CEM 2000 medium supplemented with 5% FBS, soybean trypsin inhibitor (0.1 mg/ml), carbamoylcholine (0.05 mmol), and 1% SDF-7 (Scott Laboratories). Addition of 1% SDF-7 to the culture medium provided EGF (10 ng/ml), transferrin (500 ng/ml), selenious acid (5 mg/ml), insulin (500 ng/ml), fetuin (500 ng/ml), oleic acid-bovine serum albumin (500 ng/ml), and linoleic acid-bovine serum albumin (500 ng/ml).

Primary liver tumors were induced in male ACI rats (150 g) by feeding a choline-deficient diet containing 0.2% ethionine for 12 to 16 wk, followed by a choline-sufficient diet for 8 to 12 mo (18). Liver tumors were also produced in male F-344 rats by a modification of the resistant hepatocyte protocol of Solt et al. (19). Briefly, F-344 rats (175 to 200 g) were administered a single i.p. injection of DEXA (200 mg/kg). Fourteen days after receiving the hepatocarcinogen, the selective proliferation of initiated hepatocytes was accomplished by implanting a time release pellet containing 2-AFF followed 7 days later by a two-thirds partial hepatectomy. The pellets obtained from Innovative Research of America (Gaithersburg, MD) release 2.5 mg of 2-AFF per day for 14 days. Rats were killed at 4 to 52 wk after DEXA. Liver tissues containing neoplastic foci, nodules, or primary tumors was frozen and stored as described above for normal rat tissues.

THC 1682c and THC 1677 (20) were derived from primary hepatocellular carcinoma induced in male ACI rats maintained on a choline-deficient diet containing 0.2% ethionine (Diets, Inc.), THC 252 (21, 22) was obtained from Dr. Frederick Becker (The University of Texas-M. D. Anderson Hospital and Tumor Institute, Houston, TX). Novikoff THC was derived from an aze dye-induced hepatocellular carcinoma (23). Procedures for maintenance of THC lines in vivo by s.c. or i.p. transplantation have been described previously (24).

Production of Monoclonal Antibodies. To enhance production of MAb against antigens common to THC cells, three different THC lines were used during immunization and screening. Female BALB/c mice were immunized 3 times at weekly intervals with 5 × 10^6 THC 1682c and once with 5 × 10^6 THC 1677 coated with mouse anti-normal hepatocyte antibodies. Mouse antisera against normal ACI hepatocytes was prepared as previously described (5). Three days after injection with THC 1677, spleen cells were harvested and fused with 8653 myeloma cells by the method of Kohler and Milstein (25). Hybridoma cultures showing growth in selective medium were screened by a radioimmunoassay or by a radiometric binding assay against normal hepatocytes and Novikoff THC. Two hybridomas secreting antibodies against antigens with identical mobilities on SDS-PAGE were cloned twice by limiting dilution and recloned in soft agar. The production and characterization of MAb 188.A2 and 270.38 which recognize the transferrin receptor and an oval cell-associated antigen designated OC.2, respectively, have been previously described (5, 26).

Tissue Section Analysis. Frozen sections 4 to 6 μm thick were mounted, acetone fixed, and stained with MAb as previously described (5) using affinity purified, fluorescein-conjugated, goat anti-mouse immunoglobulin obtained from Sigma. Nonspecific staining was assessed by examining tissue sections stained with culture supernatants from P3x63Ag8 myeloma cells. Sections were examined using a Nikon Microphot FX equipped with epifluorescence and a 35-mm camera. Sections were photographed with Kodak Plus X film (EI 200) and processed in Diafine developer or T-Max 100 film processed in T-Max developer.

Immunochemical Protocols. Antibodies in culture supernatants were assayed against intact, viable target cells (e.g., hepatocytes or THC) using a radiometric binding assay as previously described by Hixson and McEntire (27). Surface labeling of cells with 125I (Amersham, Arlington Heights, IL; 500 mCi/ml) was performed by the lactoperoxidase-glucose oxidase procedure of Kesk-Oja et al. (28). Cell-surface sialic acid residues were labeled as previously described (24) with 3H using the NaIO4/NaBH4 procedure described by Gahmberg and Andersson (29). Procedures for immunoprecipitation of radioiodinated antigens using Staphylococcus aureus (IgGisorb; Enzyme Center, Inc., Boston, MA) have been described (20, 24). Comparison of the reactivity of 2 different hybridoma supernatants was determined by immunodetection analysis (24). One dimensional SDS-PAGE gels were run according to the method of Laemmli (30). Two dimensional gel analysis was performed as described by O'Farrell (31). One dimensional maps of peptides generated by digestion with S. aureus V-8 protease (Miles Scientific, Naperville, IL) were prepared by the method of Cleveland et al. (32) as previously described (20). Staining, destaining, drying of gels, and autoradiography were carried out as described previously (20, 24). SDS-PAGE gels containing 35S-labeled or 1H-labeled material were impregnated with Enhance (Dupont NEN Research Products, Wilmington, DE) and visualized by fluorography (24). Apparent molecular weights were calculated from protein standards (β-galactosidase, 116,000; phosphorylase M, 97,000; bovine serum albumin, 68,000; ovalbumin, M, 43,000; Diversified Biotech, Hyde Park, MA) ran concurrently with radioactively labeled samples.

Biosynthetic Labeling. For biosynthetic labeling experiments, normal adult hepatocytes (1 × 10^6) isolated by collagenase perfusion and suspended in 2 ml of CEM 2000 supplemented with 5% FBS (Armour Pharmaceutical Co., Scottsdale, AZ), 50 μg/ml of Garamycin (Shering Corp., Kenilworth, NJ), 20 ng/ml of growth stimulator (Collaborative Research, Lexington, MA), and 1 μg/ml of insulin (Elanco Products Co., Indianapolis, IN) were placed in collagen-coated (16) 35-mm Petri dishes and allowed to attach for 30 min at 37°C. Culture medium containing unattached cells was then removed and replaced with CEM 2000 medium supplemented with 50 μM 2-mercaptoethanol (GIBCO), 1 μg/ml of insulin, and 50 μg/ml of Garamycin but without cytochrome, methionine, or FBS. After a 30-min incubation at 37°C, 1 mCi of trans-35S-label (ICN, Radiochemicals, Irvine, CA) was added, and cultures were incubated at 37°C for 18 h. Cultures were then washed 3 times in cold PBS and extracted in lysis buffer (10 mM Tris/0.15 mM NaCl/0.5% Nonidet P-40).

Deglycosylation Procedures. Digestion with endoglycosidase H (Miles Scientific), N-glycanase (peptide Nglycosidase F; Genzyme Corporation, Boston, MA), endoglycosidase F (Genzyme), and Vibrio cholerae neuraminidase (Calbiochem, La Jolla, CA) was accomplished as described McEntire et al. (33). Samples were resolved by one-dimensional SDS-PAGE.

Transplantation of Hepatocytes into the Pancreas. Host rats (200 to 225 g) were anesthetized with Metofane (Petman-Moore, Inc., Washington Crossing, NJ) and received a two-thirds partial hepatectomy (15). Immediately thereafter, the splenic portion of the pancreas was surgically exposed, and 1 × 10^6 freshly isolated or cultured hepatocytes (in a total volume of 1.0 ml of sterile PBS) were injected beneath the surface of the pancreas at multiple sites using a 26-gauge needle. The recipient rats were killed by metofane overdose 1 to 26 wk after transplantation, and the pancreas was excised and rapidly frozen as described above.

RESULTS

Immunochemical Analysis. One-dimensional and two-dimensional PAGE analysis of antigens immunoprecipitated with MAb 324.5 and MAb 324.9 from extracts of radioiodinated THC 1682c, THC 1682b, THC 252, and THC AS-30D indicated that the reactive antigens from each tumor differed in...
molecular weight, ranging from 78,000 to 92,000 (Fig. 1). On 2-dimensional gels, TuAg1 exhibited an extraordinary degree of microheterogeneity, appearing as a family of 17 to 20 spots with pIs ranging over more than 1.5 pH units (Fig. 2). Immunoprecipitation of $^3$H-labeled antigen from extracts of THC 1682c surface labeled by treatment with NaIO$_4$ and NaB$^4$H$_4$ demonstrated that TuAg1 was a sialoglycoprotein (data not shown). Digestion with peptide N-glycanase F, an enzyme which removes all N-linked oligosaccharides, produced a marked decrease in apparent molecular weight from 82,000 down to 47,000, indicating that TuAg1 on THC 1682c was more than 40% carbohydrate by weight (Fig. 1). No change in weight was detected after digestion with endoglycosidase H, endoglycosidase F, or Vibrio cholerae neuraminidase (Fig. 1).

Results from immunodepletion (Fig. 3A) and V-8 peptide analysis (Fig. 3B) demonstrated that both MAb 324.5 and MAb 324.9 were reactive with the same M, 82,000 radioiodinated peptide in extracts of THC 1682c. To determine if both MABs were also reactive with the same epitope, an aliquot of radioiodinated extract from THC 252 cells was incubated overnight with sufficient MAb 324.5 to bind all of its reactive antigen. This extract was then immunoprecipitated with MAb 324.5 or MAb 324.9 immobilized on S. aureus. As expected, preincubation with MAb 324.5 blocked precipitation with immobilized MAb 324.9, indicating that the 2 MABs are reactive with different epitopes (Fig. 3C).

Expression in Primary Cultures. Although no reactive antigen could be immunoprecipitated from extracts of freshly isolated hepatocytes, extracts of hepatocytes surface labeled with $^{125}$I after 24 h in primary culture contained readily detectable amounts of a M, 95,000 antigen reactive with MAb 324.5. This appeared to represent newly synthesized protein, since radio-labeled antigen could also be immunoprecipitated from extracts of primary hepatocytes (24-h cultures) which had been biosynthetically labeled for 2 h with $[^3]$S)methionine (Fig. 1). Similar to its THC counterpart, the hepatocyte antigen expressed both the 324.5 and 324.9 epitopes (Fig. 1).

Immunofluorescence analysis demonstrated that hepatocytes continued to express high levels of TuAg1 in culture for 7 days and that the reactivity was restricted to the cell surface (Fig. 4). Attempts to modulate the in vitro expression of TuAg1 by adding or removing various media supplements or by changing substratum composition were unsuccessful (Table 1). In addition to primary hepatocyte cultures, we also found that pancreatic acinar cells that lack TuAg1 in situ express high
Table 1. Indirect immunofluorescence analysis of acetone-fixed frozen sections obtained from adult rat tissues using MAbs 324.5 and 324.9

<table>
<thead>
<tr>
<th>Adult tissue</th>
<th>MAb 324.5</th>
<th>MAb 324.9</th>
</tr>
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<tbody>
<tr>
<td>Normal liver</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Regenerating liver (3–72 h)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Kidney</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Brain</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Intestinal mucosa and glands</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Smooth muscle</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Spleen</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Cornea</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Heart</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Tongue</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Lung</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Adrenal</td>
<td>4</td>
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</tr>
<tr>
<td>Thymus</td>
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</tr>
<tr>
<td>Trachea</td>
<td>4</td>
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<tr>
<td>Stomach</td>
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</tr>
<tr>
<td>Bladder</td>
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</tr>
<tr>
<td>Sperm</td>
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<td>4</td>
</tr>
<tr>
<td>Pancreas</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Testis</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

* MAb 324.9 reacted strongly only with small rare bundles of nerve fibers in this tissue.
* In the pancreas, MAb 324.9 reacted strongly with nerve ganglia as well as bundles of nerve fibers.
* In the testes, MAb 324.9 reacted strongly with sperm tails, an observation subsequently confirmed by examining acetone-fixed cytosmears of sperm obtained from vaginal smears.

Table 2. Effects of culture conditions on TuAg1 expression in 24-h primary rat hepatocyte cultures examined by indirect immunofluorescence analysis using MAB 324.5

<table>
<thead>
<tr>
<th>Media</th>
<th>Supplement*</th>
<th>Substratum</th>
<th>TuAg1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEM</td>
<td>10% FBS</td>
<td>RTC*</td>
<td>+</td>
</tr>
<tr>
<td>CEM/FBS</td>
<td>EGF (20 ng/ml)</td>
<td>RTC</td>
<td>+</td>
</tr>
<tr>
<td>CEM/FBS/EGF</td>
<td>TF (4 μg/ml)</td>
<td>RTC</td>
<td>+</td>
</tr>
<tr>
<td>CEM</td>
<td>1% SGF-7</td>
<td>RTC</td>
<td></td>
</tr>
<tr>
<td>CEM/FBS</td>
<td>EGF</td>
<td>Type IV collagen</td>
<td>+</td>
</tr>
<tr>
<td>CEM</td>
<td>1% SGF-7</td>
<td>Type IV collagen</td>
<td>+</td>
</tr>
</tbody>
</table>

* RTC, rat tail collagen; TF, transferrin (4 μg/ml); SGF-7, a serum replacement supplement containing insulin, EGF, TF, selenious acid, fetuin, oleic acid-bovine serum albumin, and linoleic acid-bovine serum albumin.

Fig. 4. Indirect immunofluorescence analysis of acetone-fixed primary rat hepatocytic cultures using MAb 324.5. A, 24 h, ×425; B, 72 h, ×150; and C, 168 h, ×280.

2). During fetal development, both MAb s showed reactivity with a subpopulation of liver cells on Day 14 of gestation but were unreactive with fetal liver on Days 12, 13, or 15 to 20 (Fig. 6, A and B). Pancreatic acinar cells and stomach mucosal expressions of both epitopes on Day 18 but not on Day 20 of gestation (Fig. 6, C and D). Interestingly, some cells in the capsule of the fetal stomach also showed reactivity with MAb 324.9 (Fig. 6D) but did not react with MAb 324.5. No reactivity with either MAb was observed following partial hepatectomy (3 to 72 h).

Expression of TuAg1 by Hepatic Nodules, PHC, and Lung Metastases. Although all THC lines examined in vivo were found to express TuAg1, there was concern that this might be a reflection of tumor progression or selection during transplantation rather than of an event associated with the acquisition of malignancy. However, examination by indirect immunofluorescence of PHC present in 10 rats maintained for 16 wk on choline-deficient diet containing ethionine followed by 8 to 12 mo on choline-sufficient diet showed that 11 of 14 PHC and 9 of 9 lung metastases reacted with MAb 324.5 (Fig. 7). Similar results were found when PHC were examined for expression of TuAg1 1 year after completion of the Solt/Farber protocol.
Expression of TuAg1 by Normal or Neoplastic Hepatocytes Transplanted in the Pancreas. Numerous attempts to modulate the expression of TuAg1 in primary hepatocyte cultures were unsuccessful. To determine if the in vivo microenvironment inhibited and/or modulated the expression of this oncofetal antigen in ectopically transplanted hepatocytes, freshly isolated hepatocytes (e.g., TuAg1+) or cultured hepatocytes (e.g., TuAg1−) were transplanted into the pancreas of syngeneic rats. No significant membrane expression of TuAg1 was detected in hepatocyte colonies 7 days after transplantation (Fig. 8B) even though hepatocytes from primary cultures were expressing high levels of this antigen at the time of transplantation (Fig. 9A). In contrast, staining with MAb 236.4, which recognizes a M, 110,000 normal hepatocyte cell surface antigen, showed strong membrane labeling on colonies of hepatocytes in the pancreas (Fig. 9C). Examination of pancreatic colonies of hepatocytes derived from freshly isolated hepatocytes, 6 mo after transplantation, showed that these cells continued to express normal antigens but did not express TuAg1. In contrast, when THP-252 cells were injected into the pancreas, the resulting tumor nodules continued to express high levels of TuAg1 (Fig. 9D).

DISCUSSION

Using an immunization and screening protocol designed to select for tumor-associated antigens, we have produced two hybridomas (324.5 and 324.9) defining distinct epitopes on M, 85,000 to 95,000 cell surface glycoprotein, designated TuAg1. TuAg1 is expressed on a high percentage of primary liver tumors and all metastatic lung foci and transplantable hepatic tumors examined. Immunofluorescence analysis of fetal and adult tissues showed a differential expression of the two MAb-defined epitopes. While both showed a transient expression during the development of fetal liver, pancreas, and stomach, the epitope defined by MAb 324.9 was also present on MAb 324.5-negative structures. In the adult, MAb 324.9 reactivity was restricted to sperm tails and to nerve ganglia and/or fibers in a variety of tissues. MAb 324.5, in contrast, was unreactive with all adult tissues examined. Further, neither MAb showed reactivity with regenerating rat liver following partial hepatectomy, suggesting that TuAg1 expression was linked to the differentiation stage rather than the proliferative capacity of hepatocytes. TuAg1 thus has the characteristics of an oncofetal antigen whose expression during carcinogenesis is closely associated with the acquisition of the malignant phenotype.

TuAg1 is a cell surface sialoglycoprotein with externally exposed carbohydrate and peptide moieties as shown by SDS-PAGE analysis of antigens immunoprecipitated with MAb 324.5 and 324.9 from detergent lysates of THC cells, surface labeled with 125I or with [3H] by treatment with NaIO4/NaB3H4. The apparent molecular weight of TuAg1 immunoprecipitated from THC 1683 decreased from 85,000 to 47,000 following digestion with N-glycanase. However, no detectable shift in size was noted following neuraminidase digestion, indicating a minimal artifactual contribution of sialic acid to the apparent molecular weight of the carbohydrate (35). Based on these findings and assuming an average of M, 3000 for each complex oligosaccharide moiety, it was concluded that approximately 40% of the mass of TuAg1 was contributed by 12 to 14 N-linked carbohydrate chains.

MAbs 324.5 and 324.9 were shown to be reactive with different epitopes on TuAg1 by competitive immunoprecipitation assays, immunodepletion analysis, and comparative SDS-
HEPATOCELLULAR CARCINOMA-ASSOCIATED MEMBRANE GLYCOPROTEIN

Fig. 6. Indirect immunofluorescence analysis of fetal rat tissues using MAbs 324.5 and 324.9. A, reactivity of MAb 324.5 on collagenase-dissociated 14-day fetal hepatocytes, × 600; B, paired phase-contrast for a, × 600; C, reactivity of MAb 324.5 on 18-day fetal stomach (S) and pancreas (P), × 125; D, reactivity of MAb 324.9 on 18-day fetal pancreas. Arrow denotes reactive cells in the capsule that are not detected with MAb 324.5, × 175.

Fig. 7. Immunofluorescence reactivity of MAb 324.5 on PHC (A), × 125, and lung metastasis (B), × 250, obtained from a rat fed a choline-deficient diet containing 0.2% ethionine for 16 wk, and then fed a choline-sufficient diet for an additional 8 mo.

PAGE of V-8 proteolytic fragments. This finding was consistent with the differential reactivity exhibited by the two MAbs on normal and fetal tissues. That both epitopes were exposed on the cell surface was apparent from the positive reactivity of viable intact THC cells labeled by indirect immunofluorescence with MAb 324.5 or MAb 324.9 (data not shown). Expression of the 324.5 epitope independent of the 324.9 epitope was never observed, suggesting that nonepithelial tissues, such as nerve ganglia and sperm, may express isoforms which have lost the 324.9 epitope as a consequence of tissue-specific differences in transcription or in posttranslational modifications such as phosphorylation or glycosylation. Alternatively, the antigens bearing only the 324.9 epitope may be structurally and functionally distinct from TuAg1, with homology being limited to the structural domain defined by MAb 324.9. Studies currently in progress are directed toward examining these various possibilities.

TuAg1 is distinguished from other classic oncofetal antigens such as α-fetoprotein (36) or carcinoembryonic antigen (37) by differences in size, structure, and patterns of expression in adult, neoplastic, and fetal tissues. TuAg1 also differs in these same characteristics from other neoplastic markers such as γ-glutamyltranspeptidase (38), transferrin receptor (39), placental glutathione S-transferase (40), T-6, the tumor marker described by Dunsford et al. (7, 8), HAM 6, the hyperplastic nodule-associated antigen reported by Okita et al. (41), and OFP, the cytoplasmic oncofetal protein described by Oredipe et al. (42). A relationship between TuAg1 and nerve growth factor receptor (43), a peptide which shows similarities in size and localization to the 324.9 epitope, also seems unlikely since no evidence of reactivity with TuAg1-positive THC cells was detected by indirect immunofluorescence with MAb 192-IgG, an antibody specific for the rat nerve growth factor receptor (data not shown).

Immunofluorescence and immunoprecipitation analysis of primary cultures of hepatocytes and pancreatic acinar cells revealed a high level of TuAg1 expression in 24-h cultures. The ability to immunoprecipitate 35S-labeled antigen from 24-h cultures maintained for 2 h in medium containing [35S]methionine demonstrated that this de novo expression represented newly synthesized protein rather than release of sequestered antigen. Under our standard culture conditions, hepatocytes undergo at least one round of DNA synthesis, suggesting that the induction of TuAg1 might in some way be linked to cell
Fig. 8. Indirect immunofluorescence analysis of frozen serial sections of a hepatic nodule induced by the resistant hepatocyte model (11) 28 wk after administering DENA to the rat. Reactivity of MAb 188.A2 which recognizes the transferrin receptor A, × 70; MAb 324.5 which recognizes TuaAg1 (B), × 190; and Mab 270.38 which recognizes the oval cell antigen designated OC.2 (C), × 150. Arrows denote point of reference.
cycle or proliferation. This seems unlikely, however, since TuAg1 was not detectable by indirect immunofluorescence in regenerating liver examined 3 to 72 h after partial hepatectomy. Attempts to inhibit induction or down-regulate TuAg1 expression in vitro by adding or removing various medium supplements or by changing substratum composition were unsuccessful, raising the possibility that hepatocytes in culture constitutively express this antigen. However, when positive hepatocytes from 24-h cultures were examined for expression 7 days after transplantation into the pancreas, no positive colonies were detected. In contrast, nodules or foci resulting from hepatic, pancreatic, or subcutaneous transplantation of THC continued to express high levels of antigen, demonstrating that unlike normal hepatocytes, neoplastic cells had lost the ability to regulate the expression of TuAg1.

An unexpected finding in our carcinogenesis studies was the presence of cells expressing the oval cell antigen designated OC.2 in a subpopulation of TuAg1-positive hepatic nodules present 28 wk after initiation with DENA. We have previously shown that OC.2 is associated with colonies of fetal liver cells which appear around Day 12, gradually increase in number until Day 18–19 of gestation, and then rapidly decrease until they are essentially gone by postpartum Day 14 (5, 44). TuAg1 is transiently expressed during fetal liver development (e.g., Day 14) and appears in a high percentage of PHC and lung metastases. Moreover, many of the PHC that express TuAg1 also show a heterogeneous expression of OC.2 (45). Taken together, these observations suggest that the coexpression of these antigens is associated with the appearance of fetal-like hepatocytes which have a high probability of progressing to hepatocellular carcinoma. Alternatively, the expression of TuAg1 by a subpopulation of persistent hepatic nodules may be unrelated to differentiation but instead may be a phenotypic marker for those cells which have undergone an additional step in the progression to malignancy. This latter view would be consistent with previous studies showing that only a small subpopulation of early enzyme-altered foci progress to PHC (46). In this regard, it is important to note that not all tumors present at 12 to 14 mo after DENA are positive for TuAg1. If the expression is indeed closely associated with cells committed to progress to malignancy, one would predict that TuAg1-negative tumors should lack the potential for invasion and metastasis, the hallmark behaviors of malignancy. Transplantation studies directed at testing this hypothesis are currently in progress.

In conclusion, although we do not presently understand the biological function of TuAg1, this marker will be useful for the isolation of a rare subpopulation of cells that have the neoplastic phenotype. Further characterization of these rare liver cells may ultimately allow one to delineate the cellular and molecular alterations responsible for the autonomous growth and invasiveness of malignant hepatocytes.

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Identification and Characterization of a Rat Hepatic Oncofetal Membrane Glycoprotein


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