Membrane 126-kilodalton phosphoglycoprotein associated with human carcinomas identified by a hybridoma antibody to mammary carcinoma cells

(tumors/cell surface/monoclonal antibodies)

HOWARD R. SOULE, EWERT LINDER, AND THOMAS S. EDGINGTON

Department of Immunology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, California 92037

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ABSTRACT A large number of hybridomas producing antibodies to the membrane fraction of a human mammary carcinoma cell line have been produced. Among those hybridoma antibodies that bound to exposed surface membrane epitopes on the human mammary carcinoma cell line BT-20, one has been found to bind to all tested breast carcinoma cell lines as well as to a number of other human carcinomas by solid-phase radioimmunoassay. This hybridoma antibody, designated 10-3D2, does not bind to normal mammary epithelium, a variety of control cell lines, or selected normal human tissue homogenates. The 10-3D2 hybridoma antibody identifies by immunoprecipitation a membrane 126-kilodalton protein which is indistinguishable in different tumor cells. It is glycosylated by reference to incorporation of [³H]glucosamine and also is phosphorylated by reference to incorporation of ³²P. It is present in a finely granular pattern on the cell surface of mammary carcinoma cells with concentration at the sites of apparent cell-cell contact. This 126-kilodalton phosphoglycoprotein, which we have consistently been unable to identify by immunoprecipitation of intrinsically labeled normal cells, may represent a heretofore unrecognized tumor-associated marker for human mammary carcinomas as well as certain other types of human neoplasms.

On the basis of various lines of evidence one is led to suspect that the surface of neoplastic cells may differ in one or more significant aspects from that of normal cells of the same histogenesis. Among the methods of recognition and identification of these putative differences in cell surface, it has been proposed that specific antigens are recognized by immune surveillance (1-3). It appears that specific cytolytic T lymphocytes can be induced that recognize and kill tumor cells (4), natural killer (NK) lymphocytes can recognize and attack syngeneic neoplastic cells (5, 6), protective transplantation immunity can be induced (2), information encoded in certain oncogenic viruses may be expressed at the tumor cell surface (7, 8), and extracellular proteins that associate with the normal cell may be deficient at the surface of the neoplastic derivative (9, 10). Immunologically induced protective immunity against experimental tumor transplantation and growth of spontaneously transformed cells or mutagen-transformed cells (2, 3) to be functional must probably recognize the tumor cell surface and direct effector functions to surface structures of the neoplastic cell. In spite of the abundance of circumstantial but nevertheless indirect information, discrete, characterized, and functional tumor-specific molecules immunogenic to the host remain to be well established. Among analytic approaches to the study of the plasma membrane of the neoplastic cell surface, the advent of hybridoma technology for the production of monoclonal antibodies holds promise for an expanded characterization.

Among hybridoma antibodies that identify molecules associated with human mammary carcinoma, four general types have been described. The first type of antibodies identifies constituents of the plasma membrane of the normal mammary epithelial cell, by using milk fat globules as a membrane source for immunization and selection (11-13). The second type of antibodies is derived by immunization with human mammary tumor cells, followed by selection of hybridomas on the basis of selective reaction with mammary carcinoma cells (14). The third approach has been to fuse human lymphoid cells from the lymph nodes draining a tumor to favor production of antibodies that reflect the host response to shed tumor-associated molecules (15). In a fourth approach, investigators have used hybridoma antibodies to the large tumor antigen of simian virus 40-transformed murine cells to identify a 53-kilodalton (kDal) tumor-associated phosphoprotein of human mammary carcinomas (16).

In this communication we address the identification of a trace molecule that is present on the surface of human mammary carcinomas and a number of other neoplastic cell lines but is not demonstrable on benign mammary epithelium or various non-neoplastic cells or tissues. This molecule, a 126-kDal phosphoglycoprotein, has been identified by the murine hybridoma 10-3D2, elicited by immunization with a human mammary carcinoma cell line and its isolated membranes. Unique among hybridoma antibodies that we have elicited to mammary carcinomas, it is one of only a few that appears to define a membrane molecule associated with neoplastic cells and is thus of considerable potential significance in the cell biology of the human neoplastic cell.

MATERIALS AND METHODS

Cells. Sixteen established cell lines (Table 1) were maintained as continuous adherent monolayers in Dulbecco's modified Eagle's medium (Flow Laboratories, Inglewood, CA) containing 10% fetal calf serum, 20 mM Hepes buffer, and 10 μ g gentamycin per ml. Cells were monitored regularly for mycoplasma contamination by using the fluorescent stain Hoechst 33258 and were negative. Electron microscopy and immunohistochemical localization of the 10-3D2 antigen demonstrated no mycoplasma. Normal human mammary epithelium (NHME) cells were propagated as described from primary explanted cells (18, 19). Cells were cultivated in T-175 plastic flasks and harvested with rubber policemen for preparation of cell fractions.

Generation of Hybridomas. Female BALB/c mice of 4-8 wk of age were immunized intraperitoneally with 2×10^7 BT-20 cells emulsified in complete Freund's adjuvant. Mice were given booster injections twice at 3-wk intervals with 2×10^7 viable BT-20 cells by the same route. Seven months later, the

Table 1. Cell lines utilized

Cell Line	Origin	Ref.
WI-38	Fetal human lung	17
NHME	Normal human mammary epithelium	18, 19
HBL-100	Normal human breast	20
BALB-MT1	Murine mammary carcinoma	*
NAMRU	Normal murine breast	21
M-14	Human melanoma	22
JAR-C2	Human choriocarcinoma	23
COLO-338	Human lung carcinoma	†
A-549	Human lung carcinoma	24
SK-HEP-G1	Human hepatoma	25
COLO-463	Human colon carcinoma	†
BT-20	Primary human breast carcinoma	26
AY726	Primary human breast carcinoma	27
734-B	Primary human breast carcinoma	28
MDA-MB-435-S	Pleural effusion, human metastatic breast carcinoma	29
MDA-MB-453	Pleural effusion, human metastatic breast carcinoma	29

* Unpublished data.

† G. Moore, personal communication.

mice were injected intravenously with 100 μ g of protein of a membrane fraction prepared from BT-20 cells as described (30). Four days later, spleen cells were prepared from two mice and hybridomas were prepared by fusion with SP2/0 mouse myeloma cells by conventional methods (31). After 3 days of cultivation in hypoxanthine/aminopterin/thymidine medium, cells were aliquoted at 2×10^4 cells per well in 96-well tissue culture plates (Falcon) and fed at 3- to 4-day intervals with hypoxanthine/thymidine medium (31).

Ten days after fusion, supernatants from wells with a solitary cluster of hybridoma cells were analyzed for antibody by solid-phase radioimmunoassay (SPRIA) by using 96-well round-bottomed polyvinyl chloride plates (Falcon) coated with 50 μ l of BT-20 membranes at 10 μ g/ml for 1 hr at 37°C. Plates were washed three times with saline and then were flooded with 3% bovine serum albumin/5% goat serum in saline for 1 hr to minimize nonspecific reactions. Hybridoma culture fluids were added to the assay plate (50 μ l per well) and were incubated for 3 hr at 37°C. Plates were washed four times with 140 mM NaCl/0.1% bovine serum albumin/0.05% Tween 20/10 mM sodium phosphate, pH 7.3/0.05% Na₂S₂O₃ (SPRIA buffer). Fifty microliters of immunopurified goat anti-mouse Ig labeled with ¹²⁵I (7 μ Ci/ μ g of IgG; 1 Ci = 3.7×10^{10} Bq) in SPRIA buffer was added to each well (0.3 μ Ci) and after 1 hr at 37°C, plates were washed four times with SPRIA buffer and were dried. Individual wells were detached and the radioactivity was counted. Differential specificity assays with intact cultured cells used Falcon 96-well flat-bottomed polyvinyl chloride plates that had been coated with 5×10^4 cells per well. The assay was conducted as described above.

Biosynthetic Labeling and Immunoprecipitation. Cells at 60% confluency were labeled by the addition of 50 μ Ci of [³⁵S]methionine per ml (Amersham; >1,000 Ci/mmol) in methionine-deficient Dulbecco's minimal essential medium (Irvine Scientific, Irvine, CA) for 4 hr at 37°C or were labeled similarly with 200 μ Ci of carrier-free ³²P (New England Nuclear) per ml of phosphate-free Dulbecco's minimal essential medium. Surface labeling with Na¹²⁵I (Amersham) was done by the soluble lactoperoxidase method (Sigma) as described (32). Labeling was terminated by washing cells three times with ice-cold phosphate-buffered saline (P_i/NaCl). Cells then were solubi-

lized on ice for 30 min with 1% Nonidet P-40 (NP-40) in 20 mM Tris-HCl, pH 8.3/140 mM NaCl/100 units of Trasylol per ml (3 ml per 75-cm² culture area). Extracts were clarified by centrifugation at 100,000 $\times g$ for 30 min and stored at -70°C.

One-milliliter aliquots of labeled cell extract (equivalent to $\approx 1-2 \times 10^6$ cells) each were incubated with 200 μ l of hybridoma or control tissue culture supernatant for 16 hr at 0°C. Immune complexes were collected on fixed *Staphylococcus aureus* Cowans strain 1 and washed, solubilized in NaDodSO₄/polyacrylamide gel electrophoresis sample buffer by boiling for 3 min, and analyzed by electrophoresis in 12% polyacrylamide gels in NaDodSO₄, as described by Laemmli (33). Dried gels were autoradiographed by using Kodak XAR5 x-ray film. The molecular mass markers obtained from Pharmacia were phosphorylase B (94 kDal), bovine serum albumin (67 kDal), ovalbumin (43 kDal), carbonic anhydrase (30 kDal), soybean trypsin inhibitor (20.1 kDal), and α -lactalbumin (14.4 kDal).

Immunofluorescence. Freshly trypsinized BT-20 cells were seeded onto 1-cm round glass coverslips. At subconfluence, cells were washed with P_i/NaCl and were fixed for 10 min at 4°C with a 3.5% solution of paraformaldehyde in P_i/NaCl. After washing with P_i/NaCl, cells were exposed to 0.05% NP-40 in P_i/NaCl at room temperature for 10 min to make them permeable to antibody. Cells were incubated at room temperature with a hybridoma antibody for 60 min and fluorescein-labeled goat anti-mouse immunoglobulin F(ab')₂ for 30 min. Cells were viewed by epi-illumination in a fluorescence microscope.

RESULTS

Selection and Specificity of the 10-3D2 Hybridoma Antibody. A hybridoma antibody, designated 10-3D2, was initially identified by SPRIA analysis of binding to the membrane fraction of the human mammary carcinoma cell line BT-20. Medium from the parental 10-3D2 and five clonal derivatives produced equivalent binding. To exclude reaction with tissue culture-acquired products, including mycoplasma or fetal calf serum constituents, the 10-3D2 antibody was further examined in SPRIA by using a membrane-enriched fraction from fresh tissue of an unrelated primary human mammary carcinoma. Significant binding was observed to an end point dilution of 10⁻³ (data not shown), minimizing the possibility that this antibody was binding to some nonspecific component derived from or acquired during *in vitro* cultivation of the BT-20 cell line. Further exclusion of mycoplasma was provided by the lack of demonstrable mycoplasma by fluorescent staining or immune electron microscopy.

The specificity of the 10-3D2 hybridoma antibody was assessed in a series of differential cell binding assays summarized in Fig. 1. Medium from SP2/0 myeloma culture was used as a negative control and culture supernatant from the hybridoma 20-4A2, which binds to all human cells tested, was utilized as a positive control. Binding to a cell line was statistically significant if it was greater than twice that of the negative control. Binding of 10-3D2 antibody was statistically significant, based on replicate assays, and was reproducible ($n \geq 3$). 10-3D2 antibody bound to all of five human breast carcinoma cell lines that were tested (BT-20, AY726, 734-B, MDA-MB-435S, and MDA-MB-453). Significant binding was not observed with fetal human embryonic lung cells (WI-38), tertiary culture of freshly explanted normal human mammary epithelium (NHME), a cell line derived from normal breast (HBL-100), a human hepatoma (SK-HEP-G1), and normal or malignant murine mammary gland cells (NAMRU and BALB-MT1, respectively). Significant binding by 10-3D2 was observed to human melanoma M-14, choriocarcinoma JAR-C2, lung carcinomas COLO-338 and A-549, and colon carcinoma COLO-463.

FIG. 1. Specificity of the 10-3D2 antibody determined by a differential cell-binding radioimmunoassay. Flat-bottomed 96-well polyvinyl chloride plates were coated with 5×10^4 cells per well. Procedures for binding of hybridoma antibody and detection with ^{125}I -labeled goat anti-mouse Ig are described in *Materials and Methods*.

The range of potential reaction of the 10-3D2 antibody with normal human tissues was extended by use of SPRIAs in which aqueous homogenates of human liver, stomach and small bowel, colon, lung, and spleen were employed as the immobilized antigens. This has been observed with a variety of such extracts. In addition, studies of the immunohistochemical localization of the 10-3D2-identified antigen demonstrated its absence from normal tissues (unpublished data), in contrast to the vast majority of hybridoma antibodies to breast carcinoma cells. Medium from the hybridoma 11-9H9-1B2, which binds to all human cells tested, was included as a positive control and medium from the murine myeloma cell line P3Ag8 X63.653 was included as the negative control (Table 2). Cloned hybridoma antibody 10-3D2.1 did not bind to any of the organ extracts tested, whereas the positive control 11-9H9-1B2 bound to all. The specificity of the 10-3D2 antibody is clearly not specific for only breast carcinomas as reactivity with other human carcinomas was observed.

FIG. 2. Molecular target of the 10-3D2 antibody determined by immunoprecipitation analysis. BT-20 cells were labeled biosynthetically with ^{35}S methionine (^{35}S -Met) or ^{32}P or were surface labeled *in situ* with ^{125}I and lactoperoxidase (^{125}I -LPO). NP-40-soluble cell extracts were immunoprecipitated with culture supernatant from either the 10-3D2 antibody or control supernatant from the murine myeloma SP2/0. Immune complexes were analyzed by NaDodSO₄/12% polyacrylamide gel electrophoresis and dried gels were autoradiographed. Molecular masses are shown in kDa.

Molecular Target of the 10-3D2 Hybridoma Antibody. The molecular locus for the epitope complementary to the 10-3D2 antibody was elucidated by immunoprecipitation of detergent-solubilized extracts of biosynthetically labeled cells. Autoradiographs (Fig. 2) of ^{35}S methionine-labeled BT-20 cells demonstrate a single protein of 126 kDa which was precipitated by the 10-3D2 hybridoma antibody. This protein is the substrate for a protein kinase because phosphorylation was consistently demonstrated by immunoprecipitation of extracts of ^{32}P -labeled BT-20 cells. The ^{32}P -labeled polypeptide immunoprecipitated by the 10-3D2 antibody comigrated by NaDodSO₄/polyacrylamide gel electrophoresis with the ^{35}S methionine-labeled protein precipitated by the same antibody. Because the phosphate associated with the 126-kDa protein was hydrolyzed in 1 M KOH after 2 hr at 60°C (34), we suggest that the phosphorylation site(s) may be serine or threonine, or both, rather than tyrosine (data not shown).

To determine whether the 126-kDa protein precipitated from BT-20 cell extracts is the locus of the 10-3D2 antibody-specified epitope also present in other cell lines, various human tumor

Table 2. Reactivity of 10-3D2 antibody in SPRIA against normal human tissue homogenates

¹²⁵ I-Labeled goat anti-mouse Ig, cpm bound*											
Antibody, 1:100	BT-20 membrane	Liver		GI tract		Colon		Lung		Spleen	
	1 μg	1 μg	20 μg	1 μg	20 μg	1 μg	20 μg	1 μg	20 μg	1 μg	20 μg
10-3D2.1	3,907	106	151	177	157	132	156	220	248	183	151
11-9H9.1	965	851	791	278	538	459	688	1,096	1,299	1,068	1,190
X63.653	251	92	91	166	131	98	142	238	391	123	113

Distilled water homogenates of normal human tissues were applied to assay plates as 1- μg or 20- μg protein aliquots. GI, gastrointestinal.
* The cpm bound represent the mean of three samples differing <10%. Counter background has been subtracted from each sample prior to calculation of the mean. Values shown in italics indicate a positive reaction in which cpm bound by an antibody were twice the value bound by X63.653-conditioned medium.

FIG. 3. Detection of the 10-3D2 target molecule in various human tumor cell lines. Cells were labeled biosynthetically with [^{35}S]methionine and NP-40-soluble extracts were immunoprecipitated with culture supernatant from either the 10-3D2 hybridoma or the control myeloma SP 2/0. Immunoprecipitates were analyzed by NaDodSO₄/12% polyacrylamide gel electrophoresis and dried gels were autoradiographed. Molecular masses are shown in kDa. Ca, carcinoma.

cell lines were intrinsically labeled with [^{35}S]methionine and detergent extracts were immunoprecipitated with the 10-3D2 antibody. As illustrated in selected examples in Fig. 3, it was observed that proteins identical in molecular mass to the 126-kDa protein precipitated from a BT-20 cell extract (Fig. 3, lane B) also were present in 10-3D2 immunoprecipitates of human

lung carcinoma cell line A-549 (Fig. 3, lane D) and human breast carcinoma lines MDA-MB-453 (Fig. 3, lane D) and 734-B (Fig. 3, lane H). A 126-kDa protein also was immunoprecipitated from labeled extracts of cell lines derived from human colon carcinoma COLO-463, lung carcinoma COLO-338 and melanoma (M-14), and additional breast carcinomas AY726 and MDA-MB-435-S (data not shown).

The specificity and significance of labeled proteins other than the 126-kDa species present in 10-3D2 immunoprecipitates is as yet unresolved. Some bands could represent background; however, the possibility of precursor or degradation fragments of the 126-kDa protein cannot yet be dismissed. The 126-kDa protein was reproducibly absent in extracts of WI-38 and HBL-100 cells, recapitulating specificity data obtained by SPRIA. These experiments demonstrate that the target molecules bearing the epitope for 10-3D2 antibody are similar with respect to size in various human tumor cell lines and argue against the possibility that the 126-kDa protein is characteristic of only BT-20 or even of breast carcinomas as a group. The presence of carbohydrate radicals on the 126-kDa protein also was indicated by immunoprecipitation of a molecule of identical size which was identified by autoradiography after [^3H]glucosamine labeling of cells (data not shown).

Subcellular Localization of the 126-kDa Protein. The possibility of cell surface localization of the 126-kDa molecule bearing the 10-3D2-specified epitope was assessed by surface labeling of viable BT-20 cell monolayers by lactoperoxidase-catalyzed radioiodination followed by immunoprecipitation of detergent extracts. Fig. 2 demonstrates that a single ^{125}I -labeled protein of 126 kDa was precipitated, identical in molecular mass to the [^{35}S]methionine- and ^{32}P -labeled antigens, indicating surface localization of the molecule. To confirm surface localization of the 126-kDa protein, immunohistochemical localization was performed with paraformaldehyde-fixed BT-20 cells. The 10-3D2

FIG. 4. Demonstration of surface localization of the 10-3D2 antigen by indirect immunofluorescence. BT-20 cells growing on 1-cm round glass coverslips were fixed with paraformaldehyde and made permeable to antibody with NP-40. Fixed cells then were reacted with hybridoma antibody as indicated (Left, 10-3D2; Right, 11-1H11), followed by fluorescein-labeled goat anti-mouse F(ab')₂. Stained cells were photographed by epi-illumination in a fluorescence microscope.

hybridoma antibody generated a pattern of fluorescence in a finely granular pattern along the cell periphery, especially at cell-cell junctions, indicative of a surface reaction (Fig. 4). Cells that were made permeable to antibody appeared to contain cytoplasmic 126-kDal phosphoglycoprotein antigen as well. Fig. 4 *Right* represents the immunofluorescent pattern of hybridoma antibody 11-1H11 on BT-20 cells, which reacts with all human cells tested. This figure is included to control for the peripheral staining pattern observed with the 10-3D2 antibody. Because the staining pattern in Fig. 4 *Right* is predominantly cytoplasmic, this experiment also demonstrates that the cells are permeable to antibody.

DISCUSSION

The hybridoma antibody 10-3D2, one of a panel of hybridoma antibodies derived from mice immunized with cultured human breast carcinoma cells and their membrane fractions, has been shown to be specific for an epitope residing on a cell-surface 126-kDal phosphoglycoprotein. This molecule appears to represent a rather interesting, unrecognized tumor-associated marker, in that it is associated with human mammary carcinoma cells and certain other tumor cells but not with nonneoplastic mammary epithelium or other normal or embryonic cells so far examined.

The 126-kDal protein bearing this tumor-associated epitope is conserved with respect to molecular weight, as all tumor cell lines reacting serologically with the 10-3D2 antibody contained an immunoprecipitable 126-kDal protein indistinguishable from the molecule precipitated from BT-20 cells. However, the interaction of this antibody with nonbreast tumor cells may differ from that with breast carcinoma cells. This is supported by the observation that the specific binding ratios (i.e., cpm bound to a cell line by 10-3D2 divided by cpm bound by SP2/0 culture supernatant) of 10-3D2 for the five breast carcinoma cell lines were considerably greater (13.5) compared with the other five human tumor cell lines (4.7) that demonstrated significant reactivity. The precise reason for the higher level of binding of this antibody to human breast carcinoma cells is not known, but it could reflect either a higher density of the epitope on breast tumor cells—a difference in the membrane architecture of nonbreast tumor cells resulting in modified presentation of the epitope so as to decrease the affinity of the antibody—or structural differences in the 126-kDal phosphoglycoprotein from tumor to tumor.

It has been proposed that the *in vitro* and *in vivo* diagnostic and therapeutic potential of hybridoma antibodies might require blends of various tumor-associated specificities (35). However, the use of hybridoma antibodies such as 10-3D2 that recognize a common epitope on various human tumor types might preclude the necessity of blending specificities. This is especially relevant in imaging studies in which a very high degree of specificity would be required. Initial evidence has shown that 10-3D2 is capable of imaging a human breast carcinoma growing in a nude mouse within 2 hr of administration of the antibody preparation (B. A. Khaw, personal communication). It will be of interest to determine if the broad spectrum of reactivity with various tumor types observed *in vitro* extends to these *in vivo* experiments.

Hybridoma antibodies such as 10-3D2 are being generated to delineate structural alterations of the surface of neoplastic cells, changes that may result in the malignant phenotype or may initiate and serve as the target for host immunological recognition and effector pathways. Current evidence does not allow an assignment of the 126-kDal phosphoglycoprotein to either of these groups; however, an in-depth understanding of the structure and origin of this tumor-associated marker may con-

tribute to knowledge of the molecular biology of human neoplastic cells.

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