Characterization of an Epithelial and a Tumor-associated Human Small Cell Lung Carcinoma Glycoprotein Antigen

Robert Waibel, Carl J. O’Hara, and Rolf A. Stahel

INTRODUCTION

Several groups have reported monoclonal antibodies against membrane antigens of SCC cells. Many of these antigens have been identified as glycolipids, with a large fraction directed against the lacto-N-fucopentaose III epitope. Characterization by immunoprecipitation suggested some other small cell carcinoma antigens to be proteins; these results, however, must be considered preliminary. Characterization by immunoblotting procedures have been reported on the SCC antigen MOC-1 which appears to be a protein of M, 60,000 sensitive to formalin fixation.

We have previously reported on two antibodies reacting with small cell carcinoma cell lines and tissues. By indirect immunofluorescent staining, antibody LAM2 was found to react preferentially with small cell and squamous cell carcinoma, but also with normal bronchial epithelial tissues. In contrast, the antibody LAM8 was shown to react with small cell carcinoma tissue only. Both antigens were preserved after fixation in formalin. Preliminary immunoprecipitation experiments suggested LAM2 antibody might recognize a protein and immunoblotting experiments showed LAM8 antibody to react with a glycoprotein.

This report describes the characterization of the two SCC antigens recognized by the monoclonal antibodies LAM2 and LAM8. The LAM2 antigen expression in formalin-fixed paraffin-embedded tissues was examined and compared with LAM8 expression. The glycoprotein nature of both antigens was elucidated, and the major carbohydrates of the respective epitopes were analyzed by immunoblotting and competition radioimmunoassays.

MATERIALS AND METHODS

Immunoperoxidase Staining of Tissues. Tissues fixed in alcohol-zinc-formol solution were cut in sections of 5 μm and mounted on glued slides. The sections were deparaffinized and treated with methanol and 2% swine serum as blocking reagent. The sections were covered with antibodies at dilutions of up to 1:500 and incubated for 1 h at room temperature. After washes in 0.05 M Tris buffer, pH 7.6, peroxidase-conjugated swine anti-rabbit immunoglobulin diluted 1:30 and peroxidase-conjugated swine anti-rabbit immunoglobulin diluted 1:60 (DAKO Corp., Santa Barbara, CA) were added sequentially. The reaction was localized with 3,3′-diaminobenzidine tetramonohydrate and peroxidase-conjugated swine anti-rabbit immunoglobulin diluted 1:60. Control studies included substitution with an irrelevant antibody (anti-leukocyte common antigen; DAKO).

Preparation of Monoclonal Antibodies. LAM2 and LAM8 are mouse IgM antibodies. Our procedure for antibody generation has been reported (8, 9). The antibodies were purified according to the method of Parham (10). Briefly, this was accomplished by applying the 30–55% ammonium sulfate fraction of the appropriate ascites fluid or the culture supernatant to a Sepharose 6B column (Pharmacia, Uppsala, Sweden) in PBS plus 0.5 M NaCl. The IgM positive peaks were pooled, precipitated by dialysis against 5 mM Tris, pH 7.0, and centrifugation at 45,000 × g for 1 h at 4°C. Antibody iodination. Purified LAM2 IgM was labeled with a solid phase protein iodination system (Protag-125; I. T. Baker Research, Phillipsburg, NJ) to a specific activity of 54 μCi/mg. Iodination was terminated by desalting the mixture on a small gel-filtration column (Bio-Gel P6-DG; Bio-Rad, Richmond, CA). Of the first peak eluted, 96% of the radioactivity was precipitable with trichloroacetic acid. The target uptake after labeling was determined and reached 71%.

Extraction, Enzymatic and Periodate Treatment of Antigens. For biochemical characterization the small cell carcinoma cell line SW2 (Dr. S. D. Bernal, Dana-Farber Cancer Institute) was used. Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 1 mg/ml glutamine. For Western blot analysis, cells were washed twice in PBS and solubilized in 10 mM 3-(cholamidopropyl)dimethylammonio)-1-propane sulfonate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, and 50 μg/ml pepstatin A (FLUKA, Buchs, Switzerland) in ice-cold PBS for 30 min. After centrifugation for 1 h at 130,000 × g, the supernatant was collected for further analysis. For enzymatic digestion of the cell extract, proteins were precipitated in 80% cold acetone in order to eliminate protease inhibitors. After centrifugation, the pellets were dissolved in PBS and incubated at 37°C with the following enzymes: 0.2 unit neuraminidase; 1 unit proteinase K; 1 unit chymotrypsin (tosyl lysine chloromethyl ketone); 1 unit trypsin (N-tosyl-L-phenylalanylcholoromethyl ketone) (all from Sigma); and 1 mg mixed glycosidases (mixture of exoglycosidases from Charonia lampas containing mannosidase, glucosidase, galactosidase, fucosidase, xylosidase, acetylglucosaminidase, and acetylgalactosaminidase) (Miles Scientific, Milan, Italy).

For periodate treatment of the antigens, detergent extracts were incubated with 10 mM periodate for 15 min at room temperature and the reaction was stopped with ethylene glycol.
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Immunological Detection of Antigens on Thin Layer Chromatograms. Glycolipids were isolated from SW2 cells as follows. Cell pellets (10^7 cells) were washed twice with PBS and lyophilized. The lipids were extracted using chloroform and methanol (2:1 by volume) with three times 2-min sonication. The extract was filtered through a 0.22-μm filter and evaporated on a Speed-Vac concentrator. Crude glycolipids were chromatographed on high performance thin layer chromatography silica plates (Machery-Nagel, Düren, West Germany) in chloroform:methanol:0.25% KCl, 5:4:1. After drying, the plates were either developed with ninhydrin spray reagent or immunostained as follows. The chromatograms were soaked for 2 h at room temperature in PBS and 5% nonfat dry milk to quench nonspecific binding. The plates were incubated overnight in supernatant with LAM2 or LAM8 monoclonal antibodies, washed twice for 30 min as before, and incubated with affinity-purified peroxidase-conjugated goat anti-mouse IgM(μ) (Sigma), diluted 1:500 in PBS and 10% horse serum for 2 h. After washing as before peroxidase activity was demonstrated using 4-chloro-1-naphthol as substrate.

Immunological Detection of Antigens Transferred from SDS Gels to Nitrocellulose. Electrophoresis was accomplished on 10% SDS-polyacrylamide gels under reducing conditions using the buffer system of O’Farrell (11). Fifty μg protein of the cell extracts were applied each. For LAM2 transfers, gels were first renatured 20 min each in 6 and 3 M urea and transfer buffer. Proteins were transferred electrophoretically on 0.3 μm nitrocellulose (Schleicher-Schull, Dassel, West Germany) according to the method of Towbin et al. (12) at 200 V, 1.5 A for 1 h, or 65 V for 15 h. The blotted nitrocellulose sheet was quenched by incubation (3 × 10 min) in TBS containing 5% nonfat dry milk (13). The sheet was then incubated for 16 h at room temperature with continuous rotation in LAM8, LAM2, NS-1 supernatant, or anti-Le^+ or anti-Le^− antibody (Seraclone; Biotest; Dreieich, West Germany). After washing (3 × 10 min) in TBS with 5% milk and 0.05% Tween 20 the sheets were incubated with affinity-purified peroxidase-conjugated goat anti-mouse IgM(μ) (Sigma), diluted 1:1000 in PBS and 5% milk for 1 h. After washing as before, peroxidase activity was demonstrated using 4-chloro-1-naphthol as substrate.

Radioimmunoassays. SCC cells were bound to 96-well plates as target for radioimmunoassays (14). The wells were coated with poly-L-lysine and 5 × 10^5 cells were fixed with glutaraldehyde to each well. The plates were stored at 4°C with 1% BSA and 0.2% sodium azide in PBS. For competitive inhibition of antibody binding to target cells, the optimal concentration of antibody was titrated using one-half of the maximal binding concentration. To test competition with monosaccharides, target plates were incubated with LAM2 antibody (20 μl supernatant) or LAM8 antibody (25 μl supernatant) in the presence of 100 μl 0.1 M carbohydrate solution at pH 7.0 for 1 h at room temperature. To test binding competition with lectins, target plates were preincubated with 0.1 mg lectin/well for 1 h in PBS, washed twice in PBS and 1% BSA, and incubated with the respective amount of anti LAM8 or LAM2 antibody in 100 μl PBS and 1% BSA. To test competition with human saliva, plates were incubated in the presence of 20 μl of saliva. After incubation, the plates were washed 4 times with PBS and 1% BSA, and 125I-labeled goat anti-mouse IgM immunoglobulin (0.2 μCi/50 μl) was added to each well. Each plate was again washed as before, and the individual wells were cut and counted in a gamma counter for 1 min. Typical counts for 100% binding were 60,000 cpm for LAM2 and 50,000 cpm for LAM8.

For competitive inhibition with radiolabeled LAM8 antibody, the plates were preincubated with 200 μl of supernatant of LAM2 and NS19.9 (ATCC, Rockville, MD) cultures for 1 h, followed by 5 μl (150,000 cpm) of labeled purified LAM8.

RESULTS

Expression of Antigens in Selected Tissues. The selective expression of LAM8 antigen in small cell carcinoma tissues has been reported previously. The expression of LAM2 antigen in tumor and normal tissues is summarized in Table 1 and the results compared to LAM8 expression. LAM2 and LAM8 antigens were preserved in formalin-fixed paraffin-embedded tissues. In contrast to LAM8 antigen, LAM2 was found to be expressed in lung carcinomas of every histology and adenocarcinomas of the breast, colon, and ovary. Also, LAM2 was expressed in normal bronchus, breast ducts, and the surface lining of normal ovaries, but not in normal colon. LAM2 and LAM8 were not expressed in lymphomas, sarcomas, and normal mesenchymal tissues.

Western Blot Analysis of Antigens. Initial attempts to conventionally blot LAM2 antigen after direct transfer from SDS-PAGE to nitrocellulose failed. Subsequently, Western blots made on transfers of SCC membrane extracts renatured with 6 M urea showed specific bands with approximate molecular weights between 100,000 and 120,000 and at 210,000 (Fig. 1). Conventional Western blots of SCC membrane extracts with LAM8 antibody confirmed major bands between M, 90,000 and 135,000 and revealed a second band around M, 200,000 (Fig. 2). The reactivity of neither antigen differed in gels run under nonreducing conditions.

To further elucidate the nature of the antigens membrane extracts were treated enzymatically before separation on SDS-PAGE. Treatment with periodate, chymotrypsin, and trypsin completely abolished LAM2 reactivity. Treatment with neuraminidase resulted in the disappearance of about one-half of the bands in the molecular weight range of 100,000–120,000, whereas treatment with mixed glycosidases (mixture of exoglycosidases from C. lampa containing mannosidase, glucosidase, galactosidase, fucosidase, xylosidase, acetylgalactosaminidase, and acetylglactosaminidase) resulted in the visualization of several smaller molecular weight bands below 60,000 (Fig. 1).

Specific LAM8 reactivity was lost after treatment of cell extracts or nitrocellulose filters with periodate and proteinase K. Treatment with chymotrypsin reduced the size of the high molecular band and the major bands by about M, 40,000. Binding remained unchanged after treatment with trypsin and protease K.
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205 K—« 116K—« 97K—« M 45 K—«

Fig. 1. Immunoblot of SCC extracts stained with LAM2 antibody. Molecular weight markers (M) are myosin (205,000), 8-galactosidase (116,000), phosphorylase b (97,000), bovine albumin (66,000), and egg albumin (45,000). Lanes contain untreated extract (lane 1) and extract treated with periodate (lane 2), chymotrypsin (lane 3), trypsin (lane 4), neuraminidase (lane 5), and mixed glycosidases (lane 6). K, thousands.

30 K—« 20 K—«

Fig. 2. Immunoblot of SCC extracts stained with LAM8 antibody Lane 1, untreated extract, and extract treated with periodate (lane 2), proteinase (lane 3), chymotrypsin (lane 4), trypsin (lane 5), neuraminidase (lane 6), and mixed glycosidases (lane 7). M, molecular weight markers (see legend to Fig. 1); K, thousands.

mixed glycosidases. All reactivity was lost after digestion with neuraminidase (Fig. 2).

Relationship with Blood Group Antigens. A panel of RBC expressing defined blood group antigens was incubated with antibody. Agglutination and lysis (in the presence of human AB serum) was observed with LAM2 antibody in H(O)-positive cells. LAM8 antibody had no effect on RBC with the blood group antigens A1, A2, B, O, Le*, Le*, M, and N. Antibody reactivity with saliva was examined by dot blot assays with SCC cell extracts serving as control. LAM2 recognized a component in saliva of group O secretors. LAM8 recognized a component in the saliva of Le* positive probands.

SCC cell extracts were separated on SDS-PAGE, transblotted onto nitrocellulose, and in part treated with neuraminidase. A specific band was seen with LAM8 antibody but not with anti-Le* antibody. Neuraminidase treatment of SCC extracts abolished LAM8 reactivity, but no reactivity with anti-Le* antibody emerged (Fig. 3).

Presence of Antigens in Lipid Extracts. Chloroform-methanol extracts of SCC cells were separated by thin layer chromatography and the silica gels were incubated similarly to the nitrocellulose filters. With LAM2 antibody, antigenic reactivity could be demonstrated at the chromatography front. In contrast, LAM8 antigen was not demonstrable in lipid extracts (Fig. 4).

Epitope Characterization by Competition Radioimmunoassay. SCC cells were bound to microtiter plates and antibody binding was examined by indirect radioimmunoassays. To elucidate the structure of the epitopes, antibody binding was determined after enzymatic treatment of SCC cells or in the presence of potential inhibitors. Binding of both antibodies was not influenced after treatment of target cells with mixed glycosidases. LAM2 binding was not affected by treatment with neuraminidase, while LAM8 binding was reduced to less than 10% of control.

Antibody binding was also examined in the presence of acidic mucopolysaccharides and monosaccharides (Fig. 5). Binding of
monosaccharides tested, only neuraminic acid had a marked inhibitory effect. LAM2 binding in the presence of neuraminic acid was 55% and LAM8 binding 30% of control.

A series of lectins was used to specifically block potential antibody binding sites (Fig. 6). Ulex europaeus reduced LAM2 but not LAM8 binding. This lectin recognizes α-L-fucose and blood group H antigen. Wheat germ agglutinin recognizing neuraminic acid and N-acetyl-β-D-glucosaminyl residues reduced LAM2, and to a greater extent, LAM8 binding. This effect disappeared when succinylated wheat germ agglutinin (which has no affinity to neuraminic acid) was used.

Direct radioimmunoassays with radiolabeled LAM8 antibody were performed to examine competition of other antibodies for LAM8 binding. No inhibition of LAM8 binding was observed by the monoclonal antibodies LAM2, anti-19.9, and anti-Lea.

DISCUSSION

In this paper we characterize two glycoprotein antigens of small cell carcinoma. The antigens differ in tissue expression and in molecular composition. Based on published results of immunohistochemical staining with antibodies directed against SCC membrane antigens, four major groups of antigens can be distinguished: (a) large number of antigens are also expressed in other epithelial structures, such as normal bronchial epithelium, nonsmall cell carcinoma of the lung, and nonpulmonary carcinomas such as breast or colon carcinoma. Immunohistochemical staining presented in this report showed LAM2 antigen to belong in this group of epithelial antigens, together with the antibodies SMI (15), E10/5, 2G3 (6), and PE-35 (6); (b) a second group of antigens appears to be associated with neuroendocrine differentiation. Examples include the antigen MOC-1 (7) and maybe B10/12 (6), SCLC5023 (16), and NE-35 (6). MOC-1 antigen was shown to be expressed in cells of neuroendocrine differentiation, in a small proportion of adenocarcinomas, but not in normal bronchial epithelium; (c) a third group of antigens was found to be commonly expressed in cells of macrophage differentiation or natural killer cells and SCC (17, 18); (d) a last group of antigens appears to be tumor associated, based on the absence of expression in normal bronchial epithelium; (e) a last group of antigens appears to be tumor associated, based on the absence of expression in normal bronchial epithelium; (f) a last group of antigens appears to be tumor associated, based on the absence of expression in normal bronchial epithelium; (g) a last group of antigens appears to be tumor associated, based on the absence of expression in normal bronchial epithelium.

Identification of surface proteins characteristic of small cell carcinoma by two-dimensional gel electrophoresis of radiolabeled membrane proteins has led to the hope of many investigators that their antibodies might be directed against such a protein (19). However, biochemical characterization of a large number of SCC antibodies by one group of investigators showed all their antigens to be glycolipids, many with the oligosaccharide lacto-N-fucopentaose as epitope. Our preliminary characterization of the SCC antigen LAM2 and LAM8 (8, 9) suggested the first to be a protein and the latter to be a glycoprotein antigen. The investigations reported in this communication were performed to better characterize these two antigens.

Work presented in this communication demonstrates LAM2 antigenic reactivity in crude lipid extracts and in a native confirmation glycoprotein. In immunoassays, LAM2 antigen could only be visualized after renaturation of the SCC extracts separated in SDS-PAGE. Specific bands had approximate molecular weights of 100,000–120,000 and 210,000. The antigen was sensitive to digestion with proteinases and mixed glycosi-
dases. L-Fucose was shown to be a component of the carbohydrate epitope. The epitope was shown to be related to the blood group antigen H(O) as demonstrated by the reactivity of LAM2 antibody with AB negative RBC and by inhibition of LAM2 binding of the lectin from U. europaeus.

Anti-tumor cell antibodies found to be reactive with the H-blood group determinant have been reported by other investigators. Examples in other respiratory tract tumors include antibodies against squamous cell carcinoma of the head and neck (20), lung adenocarcinoma (21), and the glycoprotein receptor for epidermal growth factor of the lung carcinoma cell line A431 (22). Earlier, a loss of AB isoantigens has been reported by immunohistochemical studies of nonsmall cell lung carcinoma with anti-AB sera (23). The uniform expression of the LAM2 antigen in SCC as well as in nonsmall cell carcinoma of the lung suggests that this phenomenon is common to lung tumors independent of their differentiation.

Immunoblotting experiments showed LAM8 antigen to be a glycoprotein with a major band at Mr 90,000-135,000 and a minor band at Mr 200,000. The antigen was sensitive to digestion with neuraminidase and chymotrypsin. Radioimmunassays showed strong competition with neuraminic acid and the lectin wheat germ agglutinin. Thus demonstrating that neuraminic acid is a major part of the LAM8 epitope. Despite reactivity of LAM8 antibody with the saliva of Le\(^+\) positive probands, several lines of investigation suggested that LAM8 antigen was different from the Le\(^+\) blood group. No LAM8 reactivity was seen with Le\(^-\) positive RBC, and SCC cell extracts did not react with anti-Le\(^+\) antibody, even after treatment with neuraminidase.

LAM8 antigen shares sensitivity to neuraminidase with other tumor-associated antigens. The most extensively characterized of these antigens is CA 19-9, a sialylated Le\(^+\) antigen first identified by a mouse monoclonal antibody raised against colon carcinoma (24). Based on the lack of Le\(^+\) reactivity of neuraminidase-treated LAM8 antigen and the lack of competition between anti-CA 19-9 and LAM8 antibody, it can be concluded that the two antigens are not identical. However, despite its relatively low molecular weight, there are suggestions that LAM8 might belong to the group of mucin antigens because of its high carbohydrate content. Evidence for this is the relatively broad specific band after separation in SDS-PAGE and the inability for radiodination of the antigen by both Bolton-Hunter or Protag methods (data not shown).

There is evidence that changes in sialylation of membrane glycoproteins might be related to the biological behavior of tumor cells. An increased membrane content of sialic acid was found to be associated with decreased transplantability of human and mouse melanomas (25), increased metastatic potential of murine tumor cells (26), and resistance to natural killer cell mediated cytolysis (27).

ACKNOWLEDGMENTS

We thank Dr. S. D. Bernal for providing the SW2 cell line and Dr. J. Gmür for performing hemagglutination experiments. We are grateful to Prof. G. Marzt for his continued support.
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