Tumor-associated Mucin-type Glycoprotein (CA54/61) Defined by Two Monoclonal Antibodies (MA54 and MA61) in Ovarian Cancers

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ABSTRACT

Two monoclonal antibodies, MA54 and MA61, were established by immunizing with culture medium supernatants of a lung adenocarcinoma cell line, and a double-determinant sandwich enzyme immunoassay system was developed by using these two monoclonal antibodies. The antigen recognized by this assay (CA54/61) was found to be often high in the sera of several cancers. The antigen recognized by MA54 (CA54) or MA61 (CA61) proved to be a carbohydrate chain on a high molecular weight mucin-type glycoprotein, and CA54 has NeuAcα2-6galactose in the terminal residue.

CA54/61 was frequently found in the sera of ovarian cancer patients, the positive rate being 67, 64, 40, and 78% in serous, mucinous, endometrioid, and mesonephroid cancers, respectively, when the cut-off value was set at mean + 4 SD. Since the positive rate of CA125, which is now the most widely used for the diagnosis of ovarian cancers, is rather low (approximately <50%) in mucinous cystadenocarcinoma, CA54/61 will be of clinical value. In addition, CA61 was detected immunohistochemically in the fetal red blood cells with nuclei, indicating its oncodevelopmental character in nature.

INTRODUCTION

Because of the difficulty in the early detection and the malignancy of human ovarian cancer, many tumor markers have recently been developed for serum diagnosis, and CA125 has been shown to have the most clinical promise (1, 2). However, the positive rate of CA125 in sera from patients with mucinous ovarian tumors has been rather low and also that of CEA2 or CA19-9, which has been reported to be useful markers for the diagnosis of mucinous cancers, has been less than 50% in many reports (3, 4). In order to overcome the disadvantages of the existing procedures, we tried to establish a new double-determinant enzyme immunoassay system with two newly developed monoclonal antibodies.

In this paper, we will report the clinical usefulness of our procedure for detecting cancer-associated antigens in sera by increasing the specificity and sensitivity, along with the preparation and the partial characterisation of the newly developed monoclonal antibodies.

MATERIALS AND METHODS

Antigen Preparation. To prepare the antigens for immunization and the enzyme immunoassay (EIA), culture supernatants of human lung adenocarcinoma cell line C1509 obtained from Dr. Kato, 1st Department of Pathology, Gifu University School of Medicine, Japan, and normal human lung fibroblast cell line CCD18Lu (ATCC No. CCL-205) grown for 2 weeks in serum-free RPMI 1640 medium were concentrated and applied to an Ultrogel AcA 44 column (IBF Bio-Technics, Villeneuve La Garenne, France) and the void fractions which were named C1509-sptAg and CCD18Lu-sptAg were collected. Protein concentration of both spt-Ags were measured by the method of Lowry et al. (5).

Monoclonal Antibodies. Monoclonal antibodies were prepared by the ordinary method. In brief, spleen cells of BALB/c mice immunized with C1509-sptAg were fused with mouse P3/X63-Ag-8.1/1 myeloma cells according to the method of Hales (6). Hybridomas selectively grown in HAT medium were screened by the indirect binding EIA. 96-well plates coated with 1 μg/well of C1509-sptAg or CCD18Lu-sptAg were incubated with the hybridoma supernatants overnight at 10°C, and then with 5 μg/ml antimouse immunoglobulins for 3 h at 25°C. Wells were then incubated with 1 μg/ml mouse peroxidase anti-peroxidase complex (The Jackson Immuno Research Laboratories Inc., West Grove, PA) for 1 h at 25°C, followed by the enzyme reaction with 0.027% H2O2-3 mg/ml O-phenylene-diamine as a substrate. Hybridomas were cloned by the limiting dilution method, and two antibodies named MA54 and MA61, which reacted with C1509-sptAg but not with CCD18Lu-sptAg, were selected.

Their immunoglobulin subclasses proved to be IgM (÷) using a MAb- ID EIA Kit (Zymed Laboratories, Inc., San Francisco, CA). Antibodies in the culture supernatants were purified by salting-out with 60% ammonium sulfate and Ultrogel AcA 22 column chromatography.

Sandwich EIA. Three sandwich EIAAs (MA54/MA54, MA54/MA61, MA61/MA61 as immobilized antibody/peroxidase-labeled antibody) were developed.

96-well plates coated with 10 μg/ml immobilized antibody (MA54 or MA61) were incubated with standard antigen (C1509-sptAg) or patient's serum which was diluted to 2% with 10% NRS-1% mouse serum (MS)-0.1% BSA overnight at 10°C. Wells were then incubated with horseradish peroxidase (HRP)-labeled (7) antibody (MA54 or MA61, 1.25 μg/ml) for 3 h at 25°C, and the enzyme reaction was performed. 50 ng of the standard antigen C1509-sptAg measured by MA54/MA61 EIA were defined as 1 unit of CA54/61. The competitive inhibition assay between MA54 and MA61 was performed by incubating simultaneously HRP-labeled MA61 (or HRP-labeled MA54) and MA54 (or MA61) in the wells in which C1509-sptAg had already been trapped by MA61 (or MA54).

Pronase Digestion. C1509-Ag was mixed with pronase (0.004 and 0.04%, Boehringer Mannheim, Mannheim, West Germany) in 200 mM Tris-HCl (pH 8.0) containing 10 mM CaCl2 and was incubated for 2 h at 37°C. The mixture was then digested by pronase and heat (0.2 or 0.3 mg/ml) and the mixture was transferred to C1509-sptAg coated wells in order to examine how the binding activity of the antigen to MA54 or MA61 was lost after the pronase digestion by the competition experiment of the indirect binding EIA between the coated antigen and the treated antigen.

Sodium Periodate Treatment. To determine whether carbohydrate was involved in the epitope of C1509-sptAg or not, C1509-sptAg was mixed with NaIO4 (0.2, 0.8, 2, and 4 mM) for 1 h at 4°C, and EIA was performed as described in the pronase digestion.

Glycosidase Treatment. C1509-sptAg-coated wells were first treated with neuraminidase (20, 100 μU/ml), α-L-fucosidase (100 μU/ml), β-galactosidase (100 μU/ml), α-N-acetylgalactosaminidase (20, 100 μU/ml in PBS, Seikagaku-Kogyo, Ltd., Tokyo, Japan), or α-N-acetylglucoaminidase (100 μU/ml in PBS, Sigma, St. Louis, MO) for 4 h at 37°C, and the indirect binding EIA was performed as described above.

NaOH Treatment. C1509-sptAg (160 U/ml) was treated with 0.05 M NaOH for 1 h at 37°C, neutralized, and then the enzyme immunoassay (EIA) was performed. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.1

1To whom requests for reprints should be addressed.
2The abbreviations used are: CEA, carcinoembryonic antigen; TLC, thin-layer chromatography; EIA, enzyme immunoassay; HRP, horseradish peroxidase.

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MUCIN-TYPE GLYCOPROTEIN (CA54/61) IN OVARIAN CANCER

Gel Filtration of CA54/61. To determine the molecular weight of CA54/61, C1509-sptAg and the sera of ovarian cancer patients were applied to an Ultrogel AcA 22 column (1.0 cm x 70 cm, equilibrated in PBS), and the antigenic activity of each fraction was measured with a MA54/MA54 or MA61/MA61 sandwich EIA.

TLC Immunostaining. Total lipids of human meconium were extracted with organic solvents and fractionated into neutral and acidic lipids by DEAE-Sephadex A-25 (Pharmacia, Upsala, Sweden) column chromatography (8). Neutral and acidic glycolipids were prepared from each fraction by acetylation, Florisil (Iwai Kagaku Co., Tokyo, Japan) column chromatography, and by saponification and dialysis, respectively, as previously described (9). Neutral and acidic glycolipids were developed on thin-layer plates (polygram; Macherey-Nagel, Duren, West Germany) with solvent systems of chloroform:methanol:water (65:35:8) and chloroform:methanol:0.5% calcium chloride in water (55:45:10) and were stained with orcinol-H2SO4 or resorcinol-HCl reagents, respectively. TLC plates were incubated with MA54 or MA61, or a monoclonal antibody Y916 which had already been proved to react specifically with NeuAcα2-6Gal structure (10), and the antibody bound to the TLC plate was detected with HRP-labeled anti-mouse IgM (Kirkegaard & Perry Lab. Inc., Gaithersburg, MD) and 4-chloro-1-naphthol as the enzyme substrate (11).

To determine a possible involvement of sialic acid as the epitope structure of monoclonal antibodies, acidic glycolipids on the plate were treated with 50 mU/ml neuraminidase (Vibrio cholerae; Behringwerke AG, Marburg, West Germany) at 37°C for 18 h.

Human Sera. The panel of sera included 92 from healthy controls, 53 from pregnant women, 91 from patients with inflammatory disease, and 57 from patients with gynecological benign tumors. Sera obtained from other malignancies included 91 from lung, 16 from pancreatic, and 57 from patients with gynecological benign tumors. Sera obtained from other malignancies included 91 from lung, 16 from pancreatic, and 57 from patients with gynecological benign tumors. Sera obtained from other malignancies included 91 from lung, 16 from pancreatic, and 57 from patients with gynecological benign tumors.

Immunoperoxidase Staining. Formalin-fixed paraffin-embedded tissue specimens were incubated with monoclonal antibodies and subsequently treated with biotinylated horse anti-mouse Ig and avidin-biotin complex reagent (Vector Laboratory Inc., Burlingame, CA). The sections were finally reacted with 0.02% diaminobenzidine and 0.02% hydrogen peroxide for 5 min at room temperature.

RESULTS

Characterization of CA54 and CA61. Two monoclonal antibodies which reacted with C1509-sptAg, but not with CDD18Lu-sptAg, were selected and named as MA54 and MA61, respectively, and their immunoglobulin class proved to be IgM (κ).

The properties of antigenic determinant recognized by MA54 (CA54) or MA61 (CA61) were tested after treatments as shown in Table 1.

The reactivities of MA54 and MA61 with C1509-sptAg were slightly reduced by pronase digestion (MA54, 17–36% loss; MA61, 12–29% loss). They were greatly diminished by sodium periodate treatment (MA54, 96–97% loss; MA61, 52–71% loss), and the degree of decrease for MA54 was greater than that for MA61. The reactivity of MA54 with C1509-sptAg was almost completely lost (91–98% loss) by the neuraminidase treatment of the antigen, and slightly decreased by α-2-acetylgalactosaminidase (4–14% loss), while the loss of reactivity of MA61 with neuraminidase was rather slight (32–45% loss) and the decrease by α-2-acetylgalactosaminidase was greater (12–27% loss) than that of MA54. The treatment of C1509-sptAg with α-2-acetylgalactosaminidase, α-L-fucosidase, and β-galactosidase showed almost no detectable change in the reactivity of both MA54 and MA61.

The reactivity of these two antibodies was completely lost by the 0.05 M NaOH-1 M NaBH₄ pretreatment of C1509-sptAg.

When C1509-sptAg and the serum of the patient with ovarian cancer were eluted by Ultrogel AcA 22 column chromatography, and the eluates were monitored by a double determinants' EIA with MA54 as the immobilized antibody and MA61 as the competitive antibody, the reactivity of HRP-labeled MA54 (or HRP-labeled MA61) with C1509-sptAg, which had already been trapped by MA54 (or MA61), was measured. In MA54/MA54 (MA61/MA61) EIA, MA61 (MA54) was added as a competitive antibody; in MA54/MA54 (MA61/MA61) EIA, MA54 (MA61) was added as a competitive antibody; in MA54/MA54 (MA61/MA61) EIA, MA22 which has already been proved not to cross-react with MA54 or MA61 (data not shown) was added as a negative control.

Immunoreactivities of MA54 and MA61 with Human Mecocoon Gangliosides. Orcinol-H₂SO₄ or resorcinol-HCl staining of acidic glycolipids indicated that human meconium contained sulfatide, G₂₂, and other minor gangliosides. TLC-immunostaining with MA54 revealed the existence of four immunoreactive gangliosides and the band with the highest mobility

Table 1 Characterization of antigen recognized by MA54 or MA61

<table>
<thead>
<tr>
<th>Enzymatic or chemical treatment of C1509-sptAg</th>
<th>Remaining reactivity after treatments</th>
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</thead>
<tbody>
<tr>
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<td>MA54</td>
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<tr>
<td>Pronase</td>
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<td>Neuraminidase</td>
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<td>α-2-acetylgalactosaminidase</td>
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<td>α-L-fucosidase</td>
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<td>β-galactosidase</td>
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<tr>
<td>Remaining reactivity after treatments</td>
<td>MA54</td>
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<td>Pronase</td>
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<tr>
<td>β-galactosidase</td>
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</table>

Fig. 1. The competitive inhibition assays between MA54 and MA61. In the MA54/MA54 (left) or MA61/MA61 (right) sandwich EIA, HRP-labeled MA54 (or HRP-labeled MA61) and MA61 (or MA54) were incubated together, and the reactivity of HRP-labeled MA54 (or HRP-labeled MA61) with C1509-sptAg, which had already been trapped by MA54 (or MA61), was measured. In MA54/MA54 (MA61/MA61) EIA, MA61 (MA54) was added as a competitive antibody; in MA54/MA54 (MA61/MA61) EIA, MA54 (MA61) was added as a competitive antibody; in MA54/MA54 (MA61/MA61) EIA, MA22 which has already been proved not to cross-react with MA54 or MA61 (data not shown) was added as a negative control.
among the MA54-positive ones was also strongly stained with antibody Y916. The reactivity of MA54 with these gangliosides was lost by the neuraminidase treatment (Fig. 2). TLC immunostaining of MA61 indicated that no component of the meconium glycolipids was reactive.

Levels of CA54/61 in Sera of Patients with Several Cancers. As shown in Fig. 3, the mean value of CA54/61 in the sera of 92 healthy controls was 8.7 U/ml, and when the cut-off value was set at 36.7 U/ml mean ± 4 SD, no case (0/92) was positive in healthy adults.

In sera of benign diseases such as inflammation, liver cirrhosis, uterine myoma, and benign ovarian cysts, the positive rates were under 15%. CA54/61 remained under the cut-off value in the sera of pregnant women throughout the entire gestational period and the sera of umbilical veins and arteries. But the value of CA54/61 was rather high in amniotic fluid. Although the positive rates for lung and pancreatic cancers were 19 and 38%, respectively, those for various cancers in liver, stomach, colon, breast, uterine cervix, and endometrium were less than 25%. Whereas, a significantly high positive rate (67%) and high mean value (164.2 U/ml) were observed for ovarian cancer patients.

Fig. 4 indicates CA54/61 levels in the sera of benign and malignant ovarian tumors. Although the positive rate of endometrioid cyst was slightly high (33%), the mean value of each benign disease was rather low. However, except for rather low levels of CA54/61 in sera from the patients with endometrioid (40%) and poorly differentiated (50%) types, the significant elevation was observed in sera of the serous type (67%, 8/12), the mucinous type (64%, 7/11), the mesonephroid type (78%, 7/9), and Krukenberg tumors (100%, 5/5).

In addition, the mean values of ovarian cancers were more than 136 U/ml except for the poorly differentiated type (26.8 U/ml).

Correlation between Serum CA54/61 and CA125 in Ovarian Cancer. Serum levels of CA54/61 and CA125 were assayed simultaneously in 29 benign ovarian tumors and 41 malignant ovarian tumors (Fig. 5).

The positive rates of CA54/61 were 68% (28/41) in ovarian cancers and 21% (6/29) in benign tumors, and those of CA125 were 78% (32/41) in ovarian cancers and 17% (5/29) in benign tumors.

When the levels of these two markers were analyzed in relation to the histological types of ovarian cancers, CA54/61 values were positive in three of five CA125-negative mucinous cystoadenocarcinoma and in two of two CA125-negative Krukenberg tumors.

No significant correlation was recognized between the serum CA54/61 and CA125 levels in ovarian cancer patients (R = 0.39; P < 0.05).

Immunohistochemical Reactivity of MA54 and MA61. By immunohistochemical technique, the staining patterns of MA54 and MA61 in cancer tissues were observed to be almost the same, but as MA61 had a tendency to stain cancer cells more specifically than MA54, the immunohistochemical reactivity of MA61 was mainly investigated to determine the localization of CA54/61.

In normal tissues, the mucus-producing glandular cells in the digestive canal, bronchus, prostate, secretory endometrium, and

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Fig. 2. TLC staining of human meconium acidic glycolipids with MA54 and Y916. Lane 1, orcinol-H$_2$SO$_4$ staining (a, $^{1}$SO$_3$-GalOseCer (sulfatide); b, $^{1}$PoNesAc-GpOseCer(Osa)); lane 2, immunostaining of acidic glycolipids with Y916; lane 3, immunostaining of acidic glycolipids with MA54; lane 4, immunostaining of acidic glycolipids with MA54 after 50 mU neuraminidase treatment.

Fig. 3. Concentration (U/ml) and positive rate (%) of serum CA54/61 in various kinds of benign diseases and cancer patients. The cut-off value was set at mean ± 4 SD (36.7 U/ml) of healthy controls. Values, mean (U/ml).

Fig. 4. Concentration (U/ml) and positive rate (%) of serum CA54/61 in benign and malignant ovarian tumors. The cut-off value was set at mean ± 4 SD (36.7 U/ml) of healthy controls. Values, mean (U/ml).

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uterine cervix were positive. In the fetus, red blood cells with a nucleus in the placental villi at 8–11-week gestation (Fig. 6) and endothelial cells of the small blood vessels in placental villi throughout the gestational period were stained with MA61.

In cancer tissues, not only adenocarcinomas of the stomach, lung, pancreas and biliary duct, but urinary bladder transitional cell carcinomas and squamous carcinomas such as skin and lung were positive.

Table 2 shows a procedure to classify the staining patterns of the MA61 in ovarian tumors. Staining patterns in each specimen were evaluated on the basis of both staining intensity and incidence of positive ovarian tumor cells. The intensity was graded on an arbitrary scale into (+), (++), (+++), and the incidence was classified into three grades (less than 10%, 10% to 50%, and more than 50%) according to the percentage of positive cells within all the tumor cells in each section. By combining both intensity and incidence, the reactivity of MA61 in each specimen was divided into weakly (W), moderately (M), and strongly (S) positive as shown in Table 3.

In benign cystoadenoma, none of the three cases of the serous type was positive, and three of four cases of the mucinous type were weakly positive.

In cystoadenocarcinoma, 54% (13–24) of the serous type (Fig. 7), 73 (8/11) of the mucinous type (Fig. 8), 67% (2/3) in the mesonephroid type and 75% (6/8) in the endometrioid type
showed a moderately or strongly positive reaction. The incidence of strongly positive cases was the highest (5/11) in mucinous cystadenocarcinoma cases, and two of two Krukenberg tumors showed a strongly positive reaction (Fig. 9).

DISCUSSION

The outline of the discussion about the epitopes (CA54 or CA61) of MA54 or MA61, and the antigen (CA54/61) recognized by both MA54 and MA61 are as follows: The sodium periodate (0.2 mm) treatment reduced the reactivity to a very small value (3%), suggesting that the antigenic determinant recognized by MA54 was composed of a carbohydrate moiety. This suggestion was also confirmed by the results of glycosidase treatments. The almost complete loss of the MA54 reactivity with neuraminidase treatment indicates that sialic acid takes a part in the carbohydrate moieties of the CA54 antigen. The fact that the reactivity of MA54 completely disappeared by 0.05 M NaOH treatment, which destroyed the O-glycoside bond, strongly suggests that the antigenic carbohydrate moiety is linked to mucin-like glycoprotein. In order to clarify the carbohydrate structure recognized by MA54, the immunoreactivity with glycolipids from meconium was investigated. The facts indicate that the antigenic determinant of MA54 or MA61 recognized by MA54 was composed of a carbohydrate moiety. This view was, as described later, supported by the immunohistochemical staining that the localization of these two monoclonal antibodies in cancer tissues resembled each other, and MA61 had a tendency to stain cancer cells more specifically than MA54.

Since the gel-filtration technique using a double-determinant assay with MA54 and MA61 revealed that the molecular weight of CA54/61 was more than \( M_c 3,000,000 \), the properties of CA54/61 could be, at present, summarized as "a high molecular weight mucin-type glycoprotein which has at least two carbohydrate epitopes, one of which has a NeuAc\( \alpha \)2-6Gal epitope in the terminal residue." Monoclonal antibody to NeuAc\( \alpha \)2-6Gal, the same epitope as MA54, has already been reported by Hakomori et al. (14) and O. Nilsson (15), and the remarkable accumulation was demonstrated in colorectal and lung carcinoma tissues. But, as CA54/61 is a glycoprotein defined with not only MA54 but MA61, and exists in the sera of cancer patients, clinical application of CA54/61 EIA can be expected. The possibility that MA54 or MA61 recognize CA125, A, B, or O blood type substances was disproved by the competitive inhibition assay with OC125 and anti-A, B, or H monoclonal antibodies (data not shown).

Common epithelial ovarian tumors such as serous, mucinous, endometrioid, and mesonephroid types constitute approximately two-thirds of all the ovarian tumors. CA125, which is now most widely used for the diagnosis of ovarian cancers, is very useful in detecting common epithelial ovarian cancers (positive rate, 80-90%) except for the mucinous type (25-50%) (1, 2). In order to overcome the shortcoming of CA125, CA19-9, and/or CEA are often used, but their positive rates in mucinous cancers are about 50% (3, 4). Therefore the development of a new tumor marker whose positive rate in mucinous cancers is more than 50% is desirable. In this respect, CA54/61 will be clinically helpful because CA54/61 was positive in 64% of mucinous cancers, even when the cut-off value was set at mean \( + 4 \) SD. Another weak point of CA125 in the diagnosis of ovarian cancers is the high false-positive rate especially in patients of pelvic endometriosis with benign endometrioid cyst (70-90%) (16). CA54/61 was positive in benign ovarian tumors in a significantly low frequency, and its positive rate in benign endometrioid cysts (33%) was lower than that of CA125. These data suggest the clinical usefulness of CA54/61 in the diagnosis of ovarian cancers and the management of postoperative patients, especially of mucinous type. Since the correlation coefficients of CA54/61 to CA125, CA19-9, or CEA were 0.39,
0.28, and -0.14 (data not shown), respectively, future combination assays of these markers are anticipated.

Since CA54/61 was defined by two monoclonal antibodies whose antigenic determinants were closely related to each other, and MA61 recognized a part of the epitope recognized with MA54, the localization of CA61 was immunohistochemically examined. As was expected, the staining patterns of MA54 and MA61 resembled each other and positive cancer cells stained with MA61 were more restricted than those with MA54. In ovarian cancers, more than 90% of the serous and mucinous types were positive, and the incidence of strong positive cases was the highest in mucinous cystoadenocarcinoma, supporting the high value of serum CA54/61 mentioned above. But, as MA61 also reacted with the normal adult tissues, transitional and squamous cell carcinoma, CA61 is not cancer specific nor adenocarcinoma specific. In fetal tissues, fetal red blood cells with nuclei in the small blood vessels of 8-11 week gestation placental villi were positive, whereas fetal blood cells of more than 12-week gestation and adult blood cells were negative, indicating that the expression of CA61 is regulated oncodevelopmentally.

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