A New Tumor-associated Antigen Useful for Serodiagnosis of Hepatocellular Carcinoma, Defined by Monoclonal Antibody KM-2

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ABSTRACT

After immunization of mice with the human hepatocellular carcinoma (HCC) cell line PLC/PRF/5, we produced monoclonal antibody KM-2, which allowed us to characterize a new HCC-associated antigen (KM-2 antigen) and to develop a sandwich-type radioimmunassay. The KM-2 antigen was strongly expressed on the cell surface of HCC cell lines. Immunofluorescence staining of frozen sections of different tissues and tumors confirmed its specific expression on the cell surface of a group of HCC. The antigen was also detected in the bile canaliculi of normal liver. Its biochemical characterization revealed a high molecular weight (Mr ~900,000) glycoprotein with an N-linked carbohydrate chain close to the peptide epitope recognized by the KM-2 monoclonal antibody. By the radioimmunassay for the KM-2 antigen, the antigen was detected in sera of 72 (47%) of 154 patients with HCC and 3 (3%) of 102 patients with liver cirrhosis. It was not detected in 96 patients with chronic hepatitis or in 100 healthy control individuals. The positive rate of KM-2 antigen was significantly higher than that of those who (51 of 154, 33%) of a-fetoprotein (AFP) when the cut-off level of AFP was taken as the widely accepted 400 ng/ml. No significant correlation was recognized between serum levels of the KM-2 antigen and AFP (r = 0.15; P > 0.05). In addition, among 103 patients with HCC whose AFP levels were less than 400 ng/ml, 31 (30%) were positive for the KM-2 antigen. Determination of the serum KM-2 antigen would be useful for the serodiagnosis of patients with HCC, particularly in cases with normal or low AFP levels.

INTRODUCTION

HCC is one of the most common malignant tumors in the world, and it has a particularly high incidence in the Far East and sub-Saharan Africa (1). Recently, hepatic imaging by such techniques as ultrasound imaging and computed axial tomography has made it possible to reliably detect small HCC lesions of less than 3 cm in diameter and in screening programs for patients with chronic liver diseases (2). Early detection of small HCC lesions has proven to be important for the treatment of patients with partial hepatectomy, and to result in better prognosis (3, 4). However, imaging techniques are not suitable for large-scale surveys of the general population. AFP is now used throughout the world for the early detection of HCC, following the finding that 69% of patients with HCC have serum AFP levels over 400 ng/ml (5, 6). However, recent reports showed that only 36% of patients with HCC had serum AFP levels over 320 ng/ml (7), and the prevalence of patients with HCC who have normal or slightly elevated AFP levels has increased (8). In addition, in one follow-up study on patients with LC who developed to HCC, patients whose AFP level exceeded 400 ng/ml accounted for only 10 of 40 cases (9). These findings point to the limits of serum AFP level in the early detection of HCC. Des-γ-carboxyprothrombin is also used for the diagnosis of HCC, and plasma levels of this marker are elevated in 55–74% of patients with HCC (10–13). However, measurement of plasma des-γ-carboxyprothrombin levels alone is not sufficient for the early detection of HCC (13). Therefore, to find an additional highly specific HCC-associated marker is still an important objective. Since the first antitumor mAb was generated (14), many attempts have been made by numerous investigators to generate mAbs that may have potential clinical applications in the diagnosis and therapy of HCC (15–22). However, no mAb has yet been generated that is useful for the serological diagnosis of HCC.

In this paper, we report a new HCC-associated antigen defined by a mAb termed KM-2, which was raised against the well-characterized human HCC cell line PLC/PRF/5 (23). We also report the development and clinical usefulness of a new sandwich-type RIA for this antigen with the use of the KM-2 mAb.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The following human cell lines were used in this study: five HCCs [PLC/PRF/5, HuH-7 (24), Hep G2 (25), Hep 3B (25), and Mahalu (26)]; one gastric carcinoma (SC-1); one colon carcinoma (COLO-205); one pancreatic carcinoma (PANC-1); one gallbladder carcinoma (G-415); one lung carcinoma (A-549); one mammary carcinoma (ZR-75-1); one melanoma (G-361); one cervix carcinoma (HeLa); two Burkitt's lymphomas (Daudi and Raji); one myeloma (GM1500); one of normal liver cells (Chang liver); one of skin fibroblasts (BUD-8); one of amniotic membrane cells (FL); and two of lung diploid fibroblasts (HAIN-55 and IMR-90). PLC/PRF/5 and Mahalu cells were the generous gift of Dr. R. H. Purcell (NIH, Bethesda, MD); HuH-7 cells were from Dr. J. Sato (Cancer Institute, Okayama University Medical School, Okayama, Japan); SC-1 and G-415 cells were from Dr. S. Koyama (University of Tsukuba School of Medicine, Tsukuba, Japan); HeLa, Raji, Chang liver, and FL cells were from Dr. S. Hayakawa (Dainabot Co., Tokyo, Japan); Daudi cells were from Dr. H. Hamaguchi (University of Tsukuba School of Medicine); GM1500 cells were from Dr. C. M. Croce (Wistar Institute, Philadelphia, PA); and HAIN-55 cells were from Dr. H. Okumura (National Institute of Health, Tokyo, Japan). Hep G2 and Hep3B, COLO-205, PANC-1, A-549, ZR-75-1, G-361, BUD-8, and IMR-90 cells were obtained from the American Type Culture Collection (Rockville, MD). PLC/PRF/5 cells were cultured in minimum essential medium (DMEM) with 45% fetal calf serum (FCS). Hepatoma cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 45% fetal calf serum (FCS) (GIBCO). Mouse SP2/0 myeloma cells and hybridomas were cultured in Dulbecco's medium.
modified Eagle’s medium containing 20% NCTC 109 medium (GIBCO), 20% FCS, 1 mm sodium pyruvate, and 0.4 unit/ml bovine insulin (Sigma Chemical Co., St. Louis, MO).

**mAb Production.** A mAb, designated KM-2, that reacted with PLC/PRF/5 cells but not with human diploid fibroblasts (HAIN-55) was obtained by fusing of SP2/0 mouse myeloma cells (27) and spleen cells of a BALB/c mouse immunized with PLC/PRF/5 cells as described by Köhler and Milstein (28) with a minor modification (29). Screening was performed by a complement-mediated cell lysis assay to obtain the HCC-associated antigen expressed on the HCC cell surface (30). Hybridomas were grown as ascites fluid in BALB/c mice using 2,6,10,14-tetramethylpentadecane (pristane) (Aldrich, Chemical Co., Milwaukee, WI), and the KM-2 mAb was purified from ascites fluid by precipitation with 50% saturated ammonium sulfate, followed by gel filtration on a column of Sephadex G-200 (Pharmacia LKB, Uppsala, Sweden) with PB, pH 7.4. Purified KM-2 mAb was used for capture and tracer antibodies for sandwich-type RIA (below). Iodination of the purified KM-2 mAb was carried out as described by Hunter and Greenwood (31). The isotype of immunoglobulin was determined by the Ouchterlony method by using a Mouse Monoclonal Typing Kit (The Binding Site Ltd., Birmingham, United Kingdom), as well as anti-mouse α and anti-mouse λ light chain antibodies (Bethyl Laboratory, Montgomery, TX). The isotype of the KM-2 mAb was IgG2a and its light chain was κ.

**Tissue and Serum Specimens.** Tissue specimens used in this study were obtained from the following sites: 20 HCCs; 14 livers adjacent to HCC; 12 livers from patients with CH; 2 livers from patients with acute hepatitis; two gastric cancers; two colon cancers; and one ovarian cancer. These tissues were obtained fresh from patients who underwent resection or needle biopsy for the respective diseases, and were stored at −80°C until use. In addition, some of normal tissues were obtained as rapidly as possible from autopsies. This study was approved by the Ethical Review Committee of the Institute of Medical Science, the University of Tokyo. Serum specimens were obtained from 154 patients with HCC, 102 patients with LC, and 96 patients with CH at the time of diagnosis, and 100 healthy control individuals. All sera were stored at −20°C until use. Patients with liver diseases were diagnosed on the basis of clinical and laboratory findings, including echography, computerized axial tomography, hepatic angiography, and/or liver history according to the criteria by Leevy et al. (32).

**Complement-mediated Cell lysis.** Complement-mediated cell lysis was determined as previously described (30). 51Cr-Labeled cells (5 × 10⁶) in 50 μl of RPMI 1640 (Nissui Co., Tokyo, Japan) containing 5% FCS were distributed in microplate wells with 50 μl of appropriate diluted ascites fluid and 50 μl of rabbit complement (1:4 dilution, Cederlane Laboratory, Ontario, Canada). After incubation for 45 min. at 37°C, the supernatant was collected from each well with a Titertek supernatant collection system (Flow Laboratories, Osaka, Japan) and counted in a gamma counter. The percentage of complement-mediated cell lysis with the KM-2 mAb was calculated by using the following formula:

\[
\text{% of cytotoxicity} = \left( \frac{\text{Test cpm} - \text{background cpm}}{\text{Maximal release cpm} - \text{background cpm}} \right) \times 100
\]

Maximal release cpm was obtained by incubating 51Cr-labeled cells with 0.05% NP-40 instead of rabbit complement.

**Reactivity of KM-2 MB with AFP, CEA, HBsAg, HbCag and Blood Cells.** To determine the reactivity of the KM-2 mAb with AFP and CEA, the KM-2 mAb, mAb to AFP (positive control), or mAb to CEA (negative control), was mixed with polystyrene beads (6.4 mm in diameter) coated with anti-mouse IgG antibody. Then each bead was incubated with AFP or CEA labeled with 125I (31), and the radioactivity on each bead was counted. Positive and negative controls gave 6027 and 228 cpm, respectively. The reactivity with CEA was tested by the same method. Positive (mAb to CEA) and negative (mAb to AFP) controls gave 2368 cpm and 80 cpm, respectively. Reactivity with HBsAg and HbCag was determined with sandwich-type RIA for anti-HBs antibody and competitive RIA for anti-HbC antibody (AUSAB and CORAB, Dainabot Co., Tokyo, Japan), respectively. Human plasmas reactive with anti-HBs antibody (positive control) and nonreactive with anti-HBs antibody (negative control) gave 8006 cpm and 90 cpm in the assay for anti-HBs antibody. Human plasmas reactive with anti-HbC antibody (positive control) and nonreactive with anti-HbC antibody (negative control) gave 660 and 24,048 cpm in the competitive assay for anti-HbC antibody, respectively. Reactivity with PBL was determined according to the method of human leukocyte antigen typing by the complement-dependent microcytotoxicity assay (33), using PBL obtained from 31 individuals with different human leukocyte antigen haplotypes. RBC were obtained from 10 individuals: 3 with blood type O, 3 with type A, 3 with type B, and 1 with type AB. After the RBC were incubated with the KM-2 mAb, the cells were stained with fluorescein isothiocyanate-conjugated anti-mouse IgG antibodies. The stained cells were analyzed by flow cy tom etry with a FACS IV (Becton Dickinson, San Jose, CA). Reactivity with blood group substances other than the ABO blood groups was determined by using a panel of RBC (Selectogen, Ortho Diagnostics Systems, Raritan, NJ) according to the manufacturer’s instructions.

**Sandwich-type RIA.** To detect the antigen recognized by the KM-2 mAb in vitro, a sandwich-type RIA was developed with the use of the KM-2 mAb. PLC/PRF/5 cells were disrupted by repeated freezing and thawing followed by centrifugation at 10,000 × g for 10 min. The supernatant was used as the assay standard. The protein concentration in the supernatant was determined by absorbance at 280 nm, and 1.0 absorbance was defined as 1 AU of the antigen. Polystyrene beads (6.4 mm in diameter) were coated with the purified KM-2 mAb, and then the beads were washed three times with PB, pH 7.4. Each bead was incubated with the PLC/PRF/5 cell supernatant. After being washed in the same manner, the beads were incubated with the 125I-labeled KM-2 mAb. Each bead was washed again and counted in a gamma counter. An assay standard curve was constructed by plotting KM-2 antigen concentrations (AU/ml) versus cpm. Experimental results were converted into AU of KM-2 antigen per ml by comparison with this standard curve.

**Solubilization and Gel Filtration of Antigen.** The supernatant of disrupted PLC/PRF/5 cells (above) was treated with 2% (v/v) of Tween-80 for 60 min at 4°C. The soluble extract was recovered by ultracentrifugation at 105,000 × g for 1 h at 4°C and the antigenic reactivity in the extract was tested by the sandwich-type RIA. The solubilized antigen solution was eluted on a column of Sephacryl S-400 (Pharmacia) with PB, pH 7.4, containing 0.05% Tween-80. Fractions were assayed for antigen activity by the sandwich-type RIA and protein concentration by absorbance at 280 nm. The column was calibrated by molecular weight markers: catalase (Mr 232,000) (Pharmacia); ferritin (Mr 440,000) (Protogen AG, Läufelfingen, Switzerland); and Blue Dextran 2000 (Pharmacia). The fractions containing the antigen recognized by the KM-2 mAb were pooled and used for determination of the biochemical characteristics below.
CaCl$_2$. The lectin solution containing 1 mg/ml of the lectin was added to an equal volume of the antigen solution. The antigenic reactivity in the mixture was assayed by the sandwich-type RIA.

**Indirect Immunofluorescence Tests.** Cultured cells and frozen tissue sections were fixed in acetone for 10 min at room temperature. The fixed cells and tissue sections were reacted with the KM-2 mAb, stained with fluorescein isothiocyanate-conjugated anti-mouse IgG antibodies (Organon Teknika Co., West Chester, PA), and observed with a fluorescence microscope (Olympus Optics Industries, Tokyo, Japan).

**Measurement of KM-2 Antigen in Sera.** The level of the KM-2 antigen was tested in serum specimens obtained from 154 patients with HCC, 102 patients with LC, and 96 patients with CH, using the sandwich-type RIA described above. The level was also tested in 100 healthy control individuals. Each serum specimen was incubated with a bead coated with the KM-2 mAb. After washing the bead, it was incubated with the $^{125}$I-labeled KM-2 mAb, washed again, and counted in a gamma counter. The level of the KM-2 antigen in the serum specimen was calculated from the standard curve, and expressed in AU/ml.

**Levels of AFP and KM-2 Antigen in Sera.** Levels of AFP in sera of 154 patients with HCC were assayed by using a commercially available RIA kit for AFP (a-FETO-RIABEAD, Dainabot). The results were expressed in ng/ml. The sera used in this assay were the same 154 serum specimens in which levels of the KM-2 antigen were determined by the sandwich-type RIA. The comparison of serum levels of AFP and the KM-2 antigen was analyzed by Student's t test and the $\chi^2$ test. A $P$ value of less than 0.05 was considered statistically significant (34). Levels of AFP in sera of 102 patients with LC and 96 patients with CH were determined by the same methods.

**RESULTS**

**Specificity of KM-2 MAb.** The reactivity of the KM-2 mAb was studied by the complement-mediated cell lysis method (Fig. 1). About 45% of $^{51}$Cr was released from PLC/PRF/5 cells or HuH-7 cells by the KM-2 mAb in the presence of rabbit complement. Little or no cell lysis was observed at any dilution of the KM-2 ascites fluid when Mahlavu cells, Chang liver cells, or human diploid IMR-90 cells were used as target cells. Hep G2 and Hep 3B cells were also killed by the KM-2 mAb in the presence of rabbit complement. All 14 of the other cell lines tested were found to be negative by the complement-mediated cell lysis assay (Table 1). All results obtained by cell lysis assay were confirmed by immunofluorescence staining (Table 1). Amounts of the antigen defined by the KM-2 mAb (hereafter referred to as KM-2 antigen) in 5 HCC cell lines and Chang liver cells were determined by sandwich-type RIA (below). The order of KM-2 antigen per wet weight of cells was: PLC/PRF/5 > Hep G2 > Hep 3B > HuH-7. Antigen was not detected in Mahlavu and Chang liver cells (data not shown). The antigen defined by KM-2 mAb was detected by indirect immunofluorescence staining with 100-fold diluted KM-2 mAb ascites fluid. The KM-2 mAb did not react with AFP, CEA, HBsAg, or HBcAg (data not shown). In addition, the KM-2 mAb was nonreactive with a panel of blood group substances tested (data not shown).

**Expression of KM-2 Antigen in Vivo.** Expression of the antigen recognized by the KM-2 mAb in vivo was further investigated by indirect immunofluorescence.

The expression of the KM-2 antigen in vivo was investigated by immunofluorescence staining of thin sections of human tumors and normal tissues with the KM-2 mAb. It was noted that the antibody reacted only with fresh-frozen sections and not with formalin-fixed and paraffin-embedded sections. Strong membranous fluorescence by the KM-2 mAb was observed with clear localization in the HCC region of liver obtained from a patient with HCC; such membranous fluorescence was not observed on liver cells adjacent to the HCC region (Fig. 2b). Interestingly, weak but significant fluorescence was observed in the bile canaliculi domain of a non-cancerous liver portion adjacent to the HCC region (Fig. 2b). Similar fluorescence was also observed in the bile canaliculi domain of normal liver (Fig. 2c). Membranous fluorescence by the KM-2 mAb was detected in 14 of 20 HCC, but not in gastric carcinoma.
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Fig. 2. Indirect immunofluorescence staining of PLC/PRF/5 HCC cells, and HCC and normal liver tissues. PLC/PRF/5 cells (a, × 400) and the frozen tissue sections (b and c, X 200) were fixed with acetone, reacted with the KM-2 mAb, and stained with fluorescein isothiocyanate-conjugated anti-mouse IgG antibody. Strong membranous staining was observed on both PLC/PRF/5 cells (a) and the HCC region in liver tissue obtained from a patient with HCC (A). Note weak but significant fluorescence (arrow) in the bile canaliculi domain of a noncancerous liver portion adjacent to the HCC region (b) and liver from a subject without liver disease (c). In c, many granules which show yellow nonspecific fluorescence are seen.

(2 cases), colon adenocarcinoma (2 cases), lung carcinoma (2 cases), or ovarian carcinoma (1 case). In addition, no membranous fluorescence was detected in the non-cancerous liver portion of 14 liver specimens obtained from patients with HCC, 12 from CH, and 2 from acute hepatitis. No staining was found on normal tissues tested: liver (2 biopsy and 4 autopsy cases), stomach (1 biopsy and 3 autopsy cases), colon (2 biopsy and 4 autopsy cases), lung (1 biopsy and 2 autopsy cases), kidney (2 biopsy and 5 autopsy cases) and pancreas (4 autopsy cases) (Table 2). Expression of the KM-2 antigen in carcinoma but not in normal tissue parts of liver obtained from HCC patients was confirmed by absorption analysis by using the paired HCC and liver adjacent to the HCC from the same patients (data not shown).

Development of Sandwich-type RIA for Antigen. We have developed a solid-phase sandwich-type RIA in which the KM-2 mAb is used both as a capture antibody and as an 125I-labeled tracer antibody. The RIA results for assay standards showed an upward linear curve from 0.3 to 10 AU/ml (Fig. 3). Intraassay coefficients of variation for the standards ranged from 2.4 to 5.0%, indicating sufficient reproducibility. The detection limit of this assay was about 0.03 AU/ml (data not shown).

Biochemical Characterization of Antigen. After treatment of the supernatant of disrupted PLC/PRF/5 cells with Tween-20 or Tween-80, the KM-2 antigen was detected in the solubilized solution. Immunoreactivity was abolished, however, by exposure of the supernatant to sodium dodecyl sulfate (0.3%), NP-40 (0.3%), or Triton X-100 (0.3%). Immunoreactivity of the antigen solubilized with Tween-80 formed a single peak on gel filtration corresponding to an approximate molecular weight of 900,000 (Fig. 4). The antigenic reactivity in this peak fraction was abolished by treatment with β-chymotrypsin (Table 3). In contrast, reactivity was enhanced markedly by treatment with N-glycanase and slightly by a mixture of O-glycanase and neuraminidase (Table 3). The addition of WGA lectin to the antigen strongly inhibited the antigenic activity in the sandwich-type RIA. The antigenic activity was less strongly inhibited also by lectins of RCA120, RCA60, and BPA (Table 4). No inhibition was observed following the addition of concanavalin A, Dolichos biflorus agglutinin, GS-I, GS-II, Lens culinaris agglutinin, Limulus polyphemus agglutinin, maclura pomifera agglutinin, PHA-Ea, PHA-L4, peanut agglutinin, soybean agglutinin, UEA-I, and UEA-II lectins (Table 4).

Table 2. Reactivity of KM-2 mAb with HCC and normal tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of cases</th>
<th>Membranous fluorescence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCC</td>
<td>20</td>
<td>+</td>
</tr>
<tr>
<td>Liver adjacent to HCC</td>
<td>14</td>
<td>+</td>
</tr>
<tr>
<td>Chronic hepatitis</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>Acute hepatitis</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Normal liver</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>Stomach</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Colon</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Lung</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Kidney</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Pancreas</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

* Reactivity of the KM-2 mAb was tested by indirect immunofluorescence assay. In seven cases, the paired HCC and normal tissue were obtained from the same patients.

**Some autopsy cases are included (see text).
between control individuals and patients with all chronic liver diseases was significantly different \( (P < 0.001) \). When a cut-off level was tentatively set at 1.0 AU/ml, which slightly exceeded the maximum level of the KM-2 antigen detected in sera of 96 patients with CH, 72 (47\%) of 154 patients with HCC and 8 (8\%) of 96 patients with LC were positive for the antigen. The cut-off value of 1.0 represented the mean ± 24.5 SD of the KM-2 antigen in 100 healthy control individuals. With the cut-off level set at 0.3 AU/ml, representing the mean ± 7 SD of the 100 healthy control sera, 120 (78\%) of 154 patients with HCC, 43 (42\%) of 102 patients with LC, and 8 (8\%) of 96 patients with CH were judged as positive for the antigen.

Serum Levels of KM-2 Antigen and AFP in Patients with HCC. Serum levels of the KM-2 antigen and AFP in 154 patients with HCC were compared (Fig. 6). The positive rate of the KM-2 antigen was 47\% (72 of 154), significantly higher than that (33\%: 51 of 154) of AFP \( (P < 0.01) \), when cut-off levels were set at 1.0 AU/ml and 400 ng/ml, respectively. Forty-one (27\%) sera were positive for both the KM-2 antigen and AFP, but 31 (20\%) were positive only for the KM-2 antigen, and 10 (6\%) were positive only for AFP. No significant correlation was recognized between the serum KM-2 antigen and AFP levels \( (r = 0.15; P > 0.05) \). In order to evaluate the ability of the KM-2 antigen to complement AFP in diagnosing HCC, levels of the KM-2 antigen were determined in patients with chronic liver diseases, including HCC, whose AFP levels were

![Graph showing standard curve for the KM-2 antigen obtained by sandwich-type RIA using the KM-2 mAb. Standard solutions (see “Materials and Methods”) were incubated with beads coated with the KM-2 mAb. The beads were washed again and counted in a gamma counter. Points, mean of triplicate samples; bars, SD.](image1)

![Graph showing gel filtration of soluble extract from PLC/PRF/5 cells with Tween-80 on a column of Sephacryl S-400 equilibrated with PB, pH 7.4, 0.05% Tween-80. The fractions were assayed for the KM-2 antigen by sandwich-type RIA. The enzymatic treatment was performed at 37°C for the number of hours indicated in parentheses. Antigenic reactivity in the reaction mixture was assayed by sandwich-type RIA.](image2)

![Table 3 Effects of enzymatic treatment on immunoreactivity of antigen recognized by KM-2 mAb](table1)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Remaining reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>β-Chymotrypsin (2 units/ml, 2 h)</td>
<td>5 ± 0.4cd</td>
</tr>
<tr>
<td>W-Glycanase (10 units/ml, 4 h)</td>
<td>303 ± 10</td>
</tr>
<tr>
<td>Mixture of O-glycanase and neuraminidase (25 μg/ml and 1 unit/ml, 4 h)</td>
<td>139 ± 4</td>
</tr>
</tbody>
</table>

*An enzyme solution was added to an equal volume of the solubilized antigen solution. The enzymatic treatment was performed at 37°C for the number of hours indicated in parentheses. Antigenic reactivity in the reaction mixture was assayed by sandwich-type RIA.*

*The percentage of remaining antigenic reactivity was calculated by using the following formula:

\[
\text{Remaining reactivity} = \frac{\text{Test cpm} - \text{background cpm}}{\text{Control cpm} - \text{background cpm}} \times 100
\]

*c After treatment with β-chymotrypsin, the enzyme reaction was inhibited by the addition of a 5% solution of bovine serum albumin and casein and a 1% solution of gelatin.

d Mean of duplicate samples ± SD.

![Table 4 Effects of lectins on immunoreactivity of antigen recognized by mAb KM-2](table2)

<table>
<thead>
<tr>
<th>Lectin</th>
<th>% of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPA</td>
<td>24 ± 2.27</td>
</tr>
<tr>
<td>RCA₄₀</td>
<td>40 ± 2.6</td>
</tr>
<tr>
<td>RCA₁₂₀</td>
<td>73 ± 5.2</td>
</tr>
<tr>
<td>WGA</td>
<td>96 ± 0.6</td>
</tr>
<tr>
<td>Con A, DBA, GS-I, GS-II, LCA, LPA, MPA, PHA-E, PHA-L, PNA, SBA, UAE-I, and UAE-II</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

* A lectin solution (1 mg/ml) was added to an equal volume of the solubilized antigen solution. The antigenic reactivity in the mixture was assayed by sandwich-type RIA.

* The percentage of inhibited antigenic reactivity was calculated by using the following formula:

\[
\text{Inhibition} = \left(1 - \frac{\text{Test cpm} - \text{background cpm}}{\text{Control cpm} - \text{background cpm}}\right) \times 100
\]

* Mean of duplicate samples ± SD.

* Con A, concanavalin A; DBA, Dolichos biflorus agglutinin; LCA, Lens culinaris agglutinin; LPA, Limulus polyphemus agglutinin; MPA, Madura pomifera agglutinin; PNA, peanut agglutinin; SBA, soybean agglutinin.
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Fig. 5. Serum levels of the KM-2 antigen. The serum levels were determined in patients with HCC, LC, and CH at the time of diagnosis, and in healthy control individuals by sandwich-type RIA using the KM-2 mAb. The cut-off value was set at 1.0 or 0.3 AU/ml. Cont., healthy control individuals.

Fig. 6. The correlation of serum levels of the KM-2 antigen and AFP in 154 patients with HCC. The dashed lines of the axes of ordinates and abscissas denote cut-off levels of the KM-2 antigen (1.0 AU/ml) and AFP (400 ng/ml), respectively. The straight line of the axis of ordinates denotes the lower cut-off level of the KM-2 antigen (0.3 AU/ml; see text). No significant correlation was recognized between the serum levels of the KM-2 antigen and AFP (r = 0.15; P > 0.05).

DISCUSSION

The KM-2 mAb reported here was reactive with 4 of 5 HCC cell lines: immunized PLC/PRF/5 cells and Hep G2, Hep 3B, and HuH-7 cells. Expression of the KM-2 antigen on the cell membranes of these cell lines was confirmed by the complement-mediated cytotoxicity assay and membranous staining by immunofluorescence assay. Other human cells including one HCC cell line, 16 cell lines, PBL from 31 individuals, and RBC from 10 individuals were found to be nonreactive with the KM-2 mAb. Thus, on limited testing of cell lines and normal cells, this antibody appears to be specific for HCC or a group of HCC, if one exists. However, immunofluorescence staining of frozen sections of different tissues and tumors revealed that the KM-2 antigen was strongly expressed on the cell surface of a group of HCC and the antigen was also detectable in the bile canaliculi domain of normal liver. The KM-2 mAb, therefore, seems to be specific for an antigen which presents in the bile canaliculi domain of liver and is abnormally expressed on cell membranes of a group of HCC. Further investigations are needed to determine existence and distribution of the KM-2 antigen in various normal and tumor tissues.

Biochemical characteristics indicate that the antigenic epitope recognized by the KM-2 antibody exists on a high molecular weight (~900,000) glycoprotein. The ability of the protease to destroy the antigenic activity seems to support the possibility that the KM-2 antibody recognizes a peptide epitope. Since the antigenic activity could be detected in detergent-solubilized and gel-filtrated fractions by a sandwich-type RIA in which the KM-2 mAb was used as the capture and tracer antibodies, two or more epitopes presumably exist on the glycoprotein. The marked enhancement of antigenic activity caused by treatment with N-glycanase suggests that carbohydrate chain(s) attached through N-glycoside linkage exist(s) near the antigenic determinant and block(s) the reaction with the KM-2 mAb. The inhibition of antigenic activity caused by the addition of WGA, RCA<sub>60</sub>, RCA<sub>120</sub>, and BPA lectins supports this possibility. Since WGA, RCA<sub>60</sub>, RCA<sub>120</sub>, and BPA lectins inhibited the immunoreactivity of the KM-2 antigen, the N-linked oligosaccharide near the determinant probably has N-acetylgalactosamine and galactose residues (35). In addition, N-acetyllactosamine possibly exists at the nonreducing end of the carbohydrate chain, because strong inhibition was observed with RCA<sub>120</sub> (36). It would be quite important to analyze biochemical characteristics of antigen preparations from different sources: normal liver and KM-2 antigen-positive sera from patients with HCC.

HCC-associated cell surface proteins or glycoproteins defined by the following mAbs have been reported: anti-PLC<sub>1</sub>, anti-PLC<sub>2</sub>, anti-PLC<sub>3</sub>, and anti-PLC<sub>4</sub> (15), P215457 and PM4E9917 (16), IBI and 9B2 (17), K-PLC2 and K-PLC3 (18), 2A3D2 and 2D11E2 (21), and Hepama-1 (22). By criteria of distribution, molecular weight (Mr 900,000) and biochemical characteristics, the KM-2 antigen seems to be different from these antigens and to be a new HCC-associated antigen. Anti-PLC<sub>1</sub> binds to an HCC cell line, Mahlavu, which was negative for the KM-2 antigen and recognizes antigens of Mr 70,000 to Mr 75,000. P215457 and PM4E9917 antibodies precipitate proteins of Mr 50,000 and Mr 65,000 from NP-40 extracts, respectively, and stain Mahlavu cells. The antigenic
determinant recognized by IB1 antibody is suggested to be carbohydrate in nature. The 9B2 antibody binds to Mahlavu cells and immunoprecipitates antigens of M1, 140,000 and M2, 130,000 from Triton X-100 cell extracts. K-PLC2 and K-PLC3 antibodies recognize antigens of M1, 200,000 and M2, 67,000, respectively, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing condition by using NP-40 extracts. 2A3D2 and 2D11E2 antibodies are directed to nonreduced carbohydrate terminus of gangliosides and sialoglycoproteins. Hepama-1 antibody reacts with an antigen of M1, 43,000 separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions by using NP-40 extracts. Although its biochemical characteristics are quite different from the KM-2 antigen, the antigen recognized by the 9B2 mAb has been detected on the bile canalicular domain of normal liver tissues (17), like the KM-2 antigen reported here. We are interested in the relationship between these antigens.

The RIA developed here would be useful for the serodiagnosis of HCC, since there was a significant difference in the serum level of the KM-2 antigen between healthy control individuals and patients with chronic liver diseases, including HCC. It was also possible to find an appropriate cut-off level to make a rough distinction between HCC and other chronic liver diseases. It should be noted, however, that the cut-off level of 1.0 AU is a tentative one, and many patients with CH and LC had levels of the KM-2 antigen between the high (1.0 AU/ml) and low (0.3 AU/ml) cut-off levels. Further efforts will have to be made to evaluate whether or not patients with chronic liver diseases who have intermediate levels of the KM-2 antigen in their sera may constitute a high risk group for HCC.

The RIA would be most useful in complementing AFP for the serodiagnosis of HCC. The finding that no significant correlation was recognized between serum levels of the KM-2 antigen and AFP may indicate that the mechanism by which the KM-2 antigen increases in the sera of patients with HCC is not related to that of AFP. Since the positive rate of the KM-2 antigen was significantly higher than that of AFP when the cut-off level of AFP was taken as 400 ng/ml (47% versus 33%; P < 0.01), the KM-2 antigen is an excellent candidate for use as an antigen for the serological diagnosis of HCC. Serum levels of AFP of more than 400 ng/ml have been accepted as a diagnostic base line for HCC, since almost all patients with LC positive for AFP have AFP values below this level. However, the prevalence of patients with HCC whose serum AFP levels are over 400 ng/ml has decreased in recent years (6–9). From this point of view it is significant that the KM-2 antigen was detected in 30% of patients with HCC with a serum AFP level of less than 400 ng/ml. Furthermore, by setting the cut-off level of the KM-2 antigen at 0.3 AU/ml instead of 1.0 AU/ml, the positive rate increased to 70% of patients with HCC, while none of the control sera exceeded this level. Although the positive rates of patients with LC and CH also add up to 42 and 8%, respectively, this lower cut-off level for the KM-2 antigen could be applied to large-scale surveys of HCC in general populations, since hepatic imaging can reliably detect small HCC lesions (2).

After finding risk groups for HCC who have high levels of either or both AFP and the KM-2 antigen, it will not be difficult to determine whether or not they have HCC. Based on these results we conclude that the KM-2 antigen would complement AFP in the diagnosis and monitoring of HCC and the RIA for the KM-2 antigen would contribute toward better prognosis by improving early detection of HCC.

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