

Molecular Identification of a Human Carcinoma-associated Glycoprotein Antigen Recognized by Mouse Monoclonal Antibody FU-MK-1

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Mouse monoclonal antibody FU-MK-1, raised against a human gastric adenocarcinoma, recognizes an antigen (termed MK-1 antigen) present on the majority of carcinomas. The present study aimed to identify the MK-1 molecule and to establish its relationship to other carcinoma antigens. Immunoprecipitation studies of human tumor cell lines revealed that FU-MK-1 recognizes a monomeric membrane glycoprotein with two forms, 40 kDa (major form) and 42 kDa (minor form), and with a molecular mass of 35 kDa following treatment with the N-glycosylation inhibitor tunicamycin. The partial amino acid sequence of a main fragment of the MK-1 molecule obtained by spontaneous cleavage under hypotonic conditions was examined, and the 17 contiguous NH₂-terminal amino acids were found to be identical with residues 81–97 of the 314-residue GA733-2 protein [Szala *et al.*; *Proc. Natl. Acad. Sci. USA*, 87, 3542–3546 (1990)]. Hence, the GA733-2 cDNA was cloned and the specificity of FU-MK-1 was confirmed using four recombinant forms of the GA733-2 antigen expressed in COS-1 cells. Immunoprecipitation with FU-MK-1 of the cell lysate transfected with the full-length GA733-2 cDNA revealed two bands corresponding to those obtained from the tumor cell lines. FU-MK-1 also precipitated three other recombinant proteins consisting of amino acids 1–265, 1–201, and 1–139 of the GA733-2 protein, respectively. Furthermore, immunoblotting analysis indicated that FU-MK-1 binds to a small fragment (6 kDa) generated from a tumor cell line under hypotonic conditions, suggesting that the FU-MK-1 epitope exists on the distal 6-kDa peptide of the extracellular domain of the GA733-2 molecule. We thus conclude that the MK-1 antigen is the GA733-2 antigen, which is currently being used as a target in clinical trials with monoclonal antibodies.

Key words: FU-MK-1 — GA733-2 — MK-1 — Monoclonal antibody — Tumor-associated antigen

Mouse monoclonal antibody (MAb) FU-MK-1, which was obtained by immunization of BALB/c mice with cancerous ascites derived from a poorly differentiated adenocarcinoma of the stomach, recognizes a tumor-associated antigen, designated MK-1 antigen, expressed on a variety of tumor tissues such as colon, gastric, pancreatic, gall bladder, bile duct, breast, and lung carcinomas.¹⁾ This antibody does not react with the normal adult gastric mucosa, but reacts weakly with the mucosa of the intestine as well as the epithelia of prostatic, mammary and thyroid glands.¹⁾ In a recent study, we developed a mouse/human chimeric antibody, designated Ch FU-MK-1, from FU-MK-1.²⁾ In combination with human lymphokine-activated killer cells, Ch FU-MK-1 showed a marked growth inhibition of human MK-1-producing tumor cells *in vitro* and in severe combined immunodeficiency mice.³⁾

The histochemical distribution of the MK-1 antigen is similar to the known distributions of a number of carcinoma-associated antigens, such as the 17-1A antigen defined by MAb CO17-1A,^{4,5)} the Trop-1 antigen^{6,7)} iden-

tified by MAb 162-21.2, the KS1/4 antigen or KSA recognized by MAb KS1/4,^{8–10)} the GA733-2 antigen specified by MAb GA733,^{11,12)} epithelial glycoprotein-2 (EGP-2) defined by MAb HEA125,^{13,14)} and the C215 antigen identified by MAb C215.^{15,16)} These antigens are expressed to varying degrees in epithelial carcinomas such as gastric, colonic, pancreatic, and mammary cancers. The antigens are also detected in lung cancers of both the non-small cell and small-cell type, so that they have been designated as the SCLC-cluster-2 antigen¹⁷⁾ at the International Workshop on Lung Tumor and Differentiation Antigens.¹⁸⁾ Recently, these antigens have been used as targets for immunotherapy using mouse antibody or mouse/human chimeric antibody in several clinical trials,^{19–23)} and a significant increase in survival as well as a reduction in the number of distant recurrences was described in patients with colorectal cancer treated with MAb CO17-1A,^{24,25)} despite the fact that the protein recognized by this antibody is also expressed to a certain degree in normal epithelial cells.²⁶⁾

In this study, we tried to identify the MK-1 antigen and to establish its relationship to other carcinoma antigens. Immunoprecipitation studies using carcinoma cell lines

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and the partial amino acid sequence analysis of the MK-1 antigen indicated immunochemical and structural similarities between the MK-1 antigen and the GA733-2 antigen.^{11, 12)} Therefore, GA733-2 cDNA was cloned from a human pancreatic carcinoma cell line, QGP-1, and used for the transfection of monkey COS-1 cells. Immunoprecipitation studies of the resultant transfected cells and immunoblotting analysis using spontaneous cleavage fragments from a tumor cell line were used for preliminary epitope mapping of FU-MK-1 on the MK-1 molecule.

MATERIALS AND METHODS

Materials Ion-exchange cellulose column "PRODUCTIV" DE was purchased from BPS Separation Ltd. (Spennymoor, UK). Immunoaffinity support AF-Tresyl TOYOPEARL 650 M was from Tosoh (Tokyo); fluorescein-conjugated affinity purified goat anti-mouse IgG antibody from Cappel (Turnhout, Belgium); alkaline phosphatase-conjugated anti-human IgG from Bio-Rad (Hercules, CA); Blocking reagent from Boehringer Mannheim (GmbH, Germany); oligo(dT)-cellulose column Oligotex-dT30 from TaKaRa Shuzo (Kyoto); [³⁵S]methionine from Amersham Life Science (Buckinghamshire, England); tunicamycin from Sigma (St. Louis, MO); First-Strand cDNA Synthesis Kit from Pharmacia Biotech (Uppsala, Sweden); pT7Blue T vector from Novagen (Madison, WI); pSG5 vector from Stratagene (La Jolla, CA); and various DNA-modifying enzymes and restriction endonucleases from Nippon Gene (Toyama) and TaKaRa Shuzo.

Monoclonal antibody Monoclonal antibody FU-MK-1 (IgG1, κ) was produced by immunizing a BALB/c mouse with cancerous ascites derived from a poorly differentiated adenocarcinoma of the stomach as described in detail by Watanabe *et al.*¹⁾ FU-MK-1 was purified from murine ascitic fluids by ion exchange chromatography on a "PRODUCTIV" DE column. A mouse myeloma protein MOPC 21 (IgG1, κ) (Organon Teknika, West Chester, PA) was used as a negative control.

Human tumor cell lines Thirty well-characterized human tumor cell lines (listed in Fig. 1) were maintained in Dulbecco's modified Eagle's medium, minimum essential medium, RPMI-1640, Ham F12K or Daigo's T medium supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μ g/ml streptomycin in 5% CO₂ at 37°C.

Flow cytometry To determine antibody binding to the cells, flow cytometry was performed by using fluorescein-conjugated goat anti-mouse IgG antibody as the tracer.²⁷⁾ The percentage of positive cells was determined using the Cell Quest computer software.

Amino acid sequencing analysis The proteins immunoprecipitated with FU-MK-1 coupled to AF-Tresyl TOYO-

PEARL were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, electrophoretically transferred to "ProBlott" polyvinylidene difluoride (PVDF) membrane (Applied Biosystems, Foster City, CA), and stained with Ponceau S. The visualized bands were excised and applied to a Pro-cise 491 protein sequencer (Applied Biosystems).

Polymerase chain reaction-based cDNA cloning Total cytoplasmic RNA was isolated from QGP-1 cells as described previously.²⁸⁾ Poly(A)⁺ RNA was isolated by passing total RNA through an oligo(dT)-cellulose column. First-strand cDNA was prepared from the Poly(A)⁺ RNA using reverse transcriptase and oligo(dT) primer in the First-Strand cDNA Synthesis Kit, and subjected as a template to polymerase chain reaction (PCR) based on 30 cycles of denaturation (at 95°C for 1 min), primer annealing (at 67°C for 1 min) and extension (at 72°C for 1.5 min) by Ampli Taq DNA polymerase on a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT). The primers designed for the amplification of the entire coding region of the GA733-2 cDNA had the following sequences: PRG-1, 5'-AAGATCTAGCATGGCGCCCCCGCAGGTC-3' and PRG-2, 5'-CAGATCTAGTTATGCATTGAGTTCCTATGCAT-3'. The primer PRG-1 hybridizes to nucleotides 105–121 in the GA733-2 cDNA¹²⁾ and contains a *Bgl*III restriction site at the 5'-end. The primer PRG-2 hybridizes to nucleotides 1029–1055 in the GA733-2 cDNA¹²⁾ and contains a *Bgl*III restriction site at the 5'-end. The resulting amplified DNA was cloned into the pT7Blue T vector and sequenced with a 373A DNA sequencer using the Fluorescent Dye Terminator sequencing kit (Applied Biosystems).

Recombinant GA733-2 genes The amplified GA733-2 cDNA was further digested with *Bgl*III and inserted into the mammalian expression plasmid vector pSG5 for expression. Three other recombinant cDNAs encoding amino acids 1–265 (Fr. 1), 1–201 (Fr. 2), and 1–139 (Fr. 3) of the GA733-2 protein, respectively, were also amplified by PCR using the cloned GA733-2 cDNA as a template. The primer PRG-1 described above was used as the 5' primer for all three recombinants. Three 3' primers, which hybridize to nucleotides 881–902, 689–710, and 506–524 in the GA733-2 cDNA and contain a *Bgl*III restriction site and a stop codon at the 5'-end, were used for amplification of the Fr. 1, Fr. 2, and Fr. 3 recombinant cDNAs, respectively. PCR amplification was performed based on 30 cycles of denaturation (at 95°C for 1 min), primer annealing (at 55°C for 1 min) and extension (at 72°C for 1.5 min) by Ampli Taq DNA polymerase, and the resultant PCR products were finally inserted into the pSG5 plasmid vector for expression.

Transfection of DNA into COS-1 cells Twenty micrograms each of plasmid DNA was transfected into 5 \times 10⁶ COS-1 cells using an electroporation apparatus (Gene Pulser, Bio-Rad, Richmond, CA) as described previ-

ously.²⁹ The transfected cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

Biosynthetic labeling and immunoprecipitation The tumor cells or transfected cells were cultured for 6 h with 1.85 MBq/1.5 ml of [³⁵S]methionine in methionine-free minimum essential medium in the presence or absence of 5 μ g/ml tunicamycin. The labeled cells were washed twice with phosphate-buffered saline (PBS) and lysed with a detergent-containing buffer (PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and proteinase inhibitors). After centrifugation at 15,000g for 10 min, radiolabeled antigens in the supernatants were immunoprecipitated by incubation with FU-MK-1 coupled to AF-Tresyl TOYOPEARL, and then analyzed by SDS-PAGE followed by fluorography.³⁰

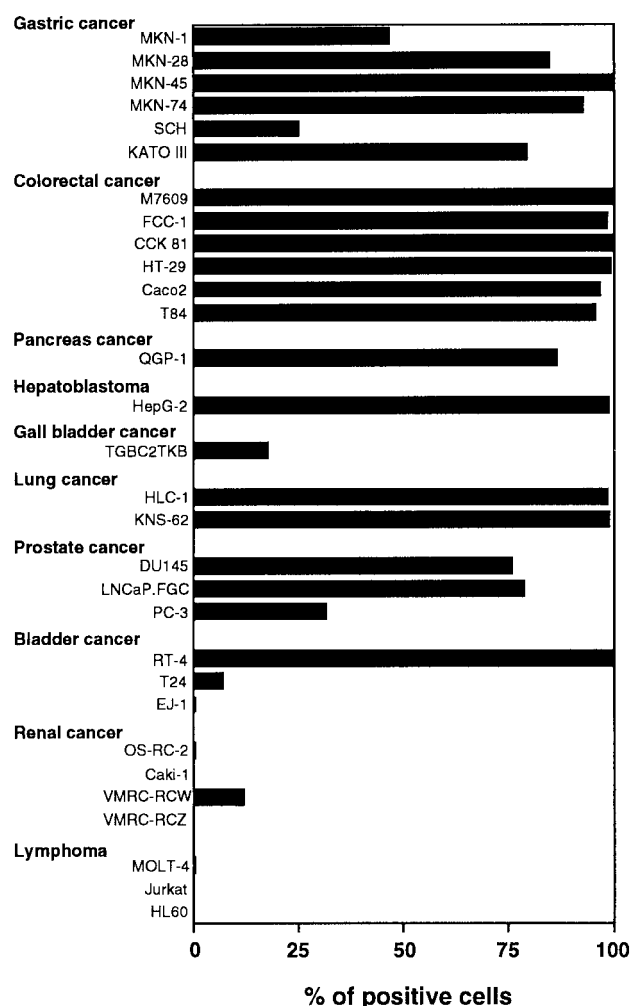


Fig. 1. Reactivity of FU-MK-1 with various human tumor cell lines determined by flow cytometry. About 1×10^4 cells of each cell line were analyzed and the positive cells were determined using the Cell Quest computer software.

Immunoblotting The tumor cell lines were also lysed under a hypotonic condition (see below). The immunoprecipitated proteins with FU-MK-1 coupled to AF-Tresyl TOYOPEARL were separated by SDS-PAGE (15% gels) under reducing or non-reducing conditions and transferred onto a PVDF membrane sheet (Millipore, Bedford, MA). The membrane sheet was incubated with a blocking buffer containing T-TBS (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20) and 0.5% Blocking reagent followed by incubation with a mouse/human chimeric antibody to MK-1 (Ch FU-MK-1)² diluted with the blocking buffer. After being washed with T-TBS, the membrane sheet was incubated with alkaline phosphatase-conjugated anti-human IgG diluted with the same blocking buffer. The immunoreactive proteins were visualized by enzyme reaction with the substrate 5-Br-4-Cl-3-indolyl phosphate and nitro-blue tetrazolium.

RESULTS

Reactivity of FU-MK-1 with various human tumor cell lines in flow cytometry Since it has been found that FU-MK-1 histochemically reacts with a variety of tumor tissues,¹ we also tested the reactivity of this antibody with various tumor cell lines. As shown in Fig. 1, FU-MK-1 bound to cells of all gastrointestinal tumors tested (stomach, colon, pancreas, liver, and gall bladder). In addition, the MAb bound to cells of carcinomas of the lung (2/2)

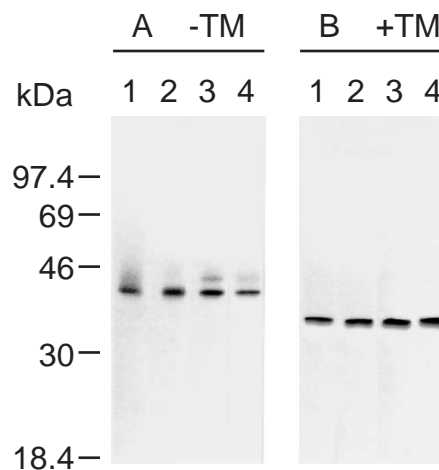


Fig. 2. Detection of the FU-MK-1 target molecule synthesized by various carcinoma cell lines. The tumor cells were metabolically labeled with [³⁵S]methionine for 6 h in the absence (A) or presence (B) of 5 μ g/ml tunicamycin (TM). Immunoprecipitates with FU-MK-1 from the detergent cell lysates were separated by SDS-PAGE (10% gel) under reducing conditions with 2-mercaptoethanol. Cell lines used (A and B): lane 1, MKN-74; lane 2, QGP-1; lane 3, HLC-1; and lane 4, CCK-81.

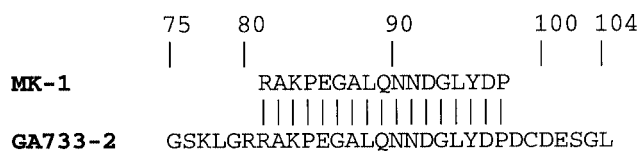


Fig. 3. Sequence comparison between the amino-terminal sequence of the MK-1 antigen and the GA733-2 antigen. The upper sequence shows the partial amino acid sequence obtained from the MK-1 antigen, and the lower sequence depicts amino acids 75 through 104 of the GA733-2 protein sequence.

and prostate (3/3). Of the 3 bladder carcinoma cell lines, two were reactive with this MAb and only one of the 4 renal cell lines was slightly reactive with this MAb. While this MAb did not react with all 3 lymphoma cell lines tested. Thus, FU-MK-1 reacted with most carcinoma cell lines tested.

Immunoprecipitation of MK-1 antigen synthesized by tumor cells Four representative cell lines (MKN-74 of gastric origin, QGP-1 of pancreatic origin, HLC-1 of lung origin, and CCK-81 of colonic origin) were metabolically labeled with [³⁵S]methionine and their cell lysates were submitted to immunoprecipitation with FU-MK-1 followed by SDS-PAGE and fluorography. As shown in Fig. 2A, HLC-1 and CCK-81 cells gave rise to two components with molecular masses of 40 and 42 kDa, and MKN-74 and QGP-1 cells yielded one band with a molecular mass of 40 kDa. When treated with the N-glycosylation inhibitor tunicamycin, all four tumor cell lines produced a single band with a molecular mass of 35 kDa (Fig. 2B), indicating that two bands of HLC-1 and CCK-81 may be caused by different degrees of glycosylation and that the epitope recognized by FU-MK-1 is protein in nature.

Amino acid sequence of MK-1 antigen The MK-1 antigen was immunoprecipitated from the detergent extract of MKN-45 cells by FU-MK-1 coupled to AF-Tresyl TOYOPEARL. The eluted material was subjected to SDS-PAGE. Two bands of 40 and 42 kDa were observed by fluorography after biosynthetic labeling. An attempt was made to sequence the 40- and 42-kDa species, but no phenylthiohydantoin derivatized amino acids were identified, suggesting a blocked amino terminus. When we tried to purify the MK-1 antigen, we found that after incubation of living tumor cells in a hypotonic buffer (5 mM Hepes, pH 7.4, containing 50 mM mannitol and 10 mM CaCl₂), a main band of 34 kDa appeared specifically in SDS-PAGE under reducing conditions followed by staining with Ponceau S (data not shown). A 17-amino-acid sequence of the smaller 34-kDa form was identified (Fig. 3). The search for homologous sequences in the SWISSPROT protein sequence data base revealed high homology of the above sequence with that of the cloned carcinoma-associated

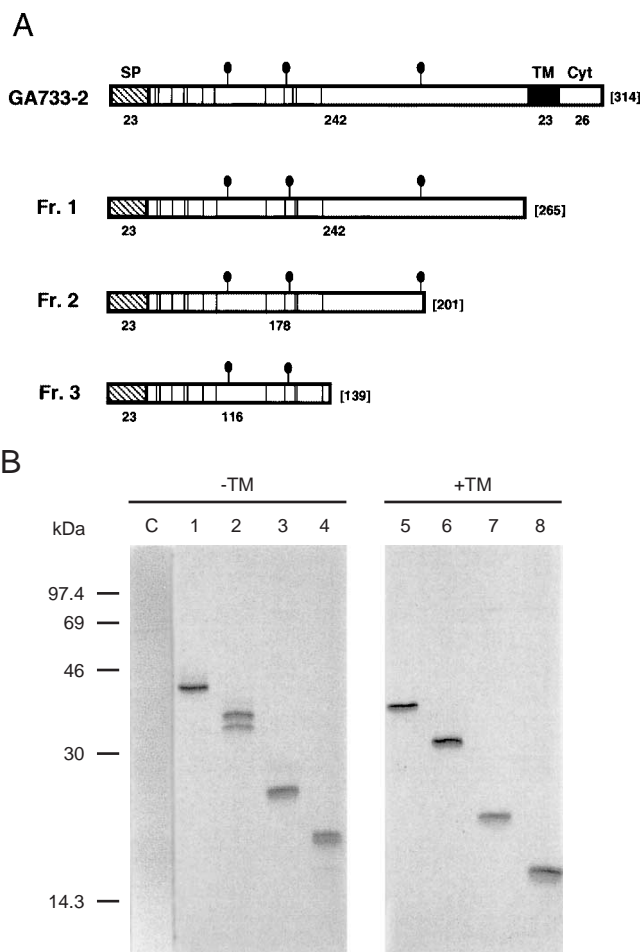


Fig. 4. (A) The abbreviated names and schematic structures of GA733-2 and three recombinant GA733-2 proteins used in this study. Vertical lines drawn in the protein backbone (open bars) indicate cysteine residues. Potential N-linked glycosylation sites are shown as black dots attached to the protein backbone. SP, cleavable signal peptide which is not present on the mature-type membrane protein; TM, transmembrane domain; Cyt, cytoplasmic domain. Amino acid numbers reconstructed in this study are presented below the blocks. The numbers in brackets show the total numbers of amino acids of the respective recombinants. (B) Reactivity of FU-MK-1 with GA733-2 antigen and GA733-2 fragments expressed in COS-1 transfectant cells. The transfectant cells were metabolically labeled with [³⁵S]methionine for 6 h in the absence (-TM) or presence (+TM) of 5 μg/ml tunicamycin (TM). Immunoprecipitates with FU-MK-1 from detergent cell lysates were separated by SDS-PAGE (10% gel) under reducing conditions with 2-mercaptoethanol. Cell lines used: lane C, parental COS-1; lanes 1 and 5, GA733-2; lanes 2 and 6, Fr. 1; lanes 3 and 7, Fr. 2; and lanes 4 and 8, Fr. 3.

antigen GA733-2¹²): the 17 identified contiguous amino-terminal residues of the MK-1 antigen are identical with amino acids 81–97 of the GA733-2 protein (Fig. 3).

Molecular cloning and expression of recombinant GA733-2 cDNAs In order to determine further whether the MK-1 antigenic epitope is encoded within the 314-amino-acid GA733-2 protein, four recombinant forms of this antigen were expressed in COS-1 cells (Fig. 4A). To obtain cDNA encoding GA733-2, cytoplasmic mRNA was purified from QGP-1 cells and used as a template for PCR after single-strand cDNA synthesis. One pair of oligonucleotide primers was used so that the resulting PCR fragment would cover the entire coding sequence of the GA733-2 cDNA. The resulting amplified DNA was analyzed by electrophoresis on a 0.8% agarose gel and found to consist of a single product of the expected size (965 base pairs). After cloning into the pT7Blue T plasmid vector, the DNA sequence was determined and the region encoding the GA733-2 open reading frame was found to be identical to that reported by Szala *et al.*¹²⁾ To derive mammalian cells expressing the cDNA that encodes the GA733-2 antigen, the amplified cDNA was digested with *Bgl*III and inserted into the expression plasmid vector pSG5. The resulting expression vector was then transfected into COS-1 cells.

Three other recombinant cDNAs encoding amino acids 1–265 (Fr. 1), 1–201 (Fr. 2), and 1–139 (Fr. 3) of the GA-

733-2 protein, respectively (Fig. 4A), were also amplified. When analyzed by electrophoresis on 0.8% agarose gel, the resulting amplified DNAs each showed a single band with the expected size (813 base pairs for Fr. 1, 621 base pairs for Fr. 2, and 435 base pairs for Fr. 3) (data not shown). The resultant PCR products were inserted into the pSG5 plasmid vector for expression and transfected into COS-1 cells.

Reactivity of FU-MK-1 with recombinant GA733-2 antigens expressed in COS-1 cells The epitope of FU-MK-1 was preliminarily mapped by immunoprecipitation analysis of the recombinant GA733-2 proteins. Immunoprecipitation with FU-MK-1 of COS-1 cells transfected with the full-length GA733-2 cDNA revealed a band with a molecular mass of 40 kDa (Fig. 4B, lane 1). This appeared at the place corresponding to each major band from MKN-74, QGP-1, HLC-1, and CCK-81 (Fig. 2A, lanes 1–4). The band of the transfected COS-1 cells migrated as a single band with a molecular mass of 35 kDa after treatment with tunicamycin (Fig. 4B, lane 5), and this value is in agreement with those of the bands obtained from the four tumor cell lines by tunicamycin treatment (Fig. 2B, lanes 1–4). In the parental COS-1 cells, no specific band was seen with FU-MK-1 (Fig. 4B,

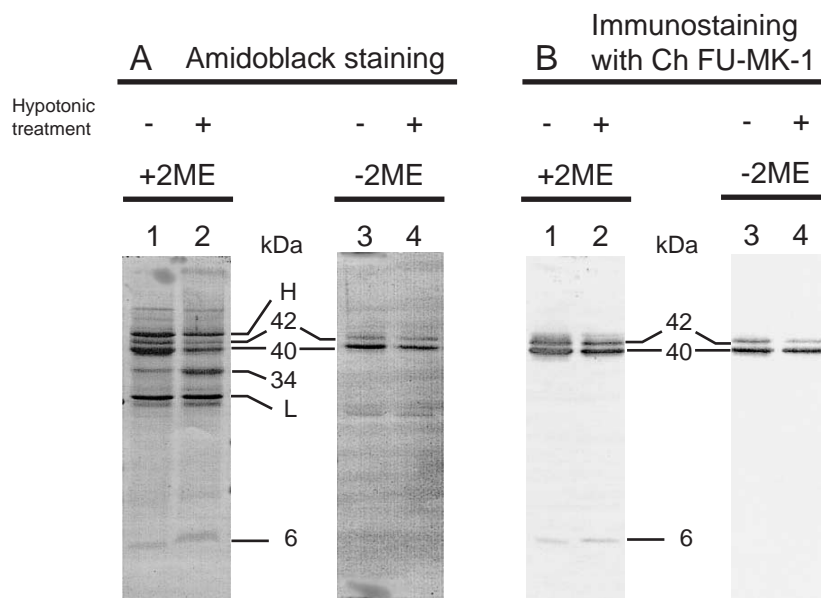


Fig. 5. Characterization of antigenic determinants by immunoblotting. The MK-1 antigen was immunoprecipitated with FU-MK-1 from MKN-45 cells with (+) or without (-) hypotonic treatment. The immunoprecipitates were resolved by SDS-PAGE (15% gel) under reducing conditions with (+) or without (-) 2-mercaptoethanol (2ME), and then blotted on PVDF membrane. The protein bands were detected by Amidoblack staining (A) or immunoblotting (B). In the immunoblotting, the MK-1 proteins were detected by successive incubation with a mouse/human chimeric antibody (Ch FU-MK-1) to MK-1, with alkaline phosphatase-conjugated anti-human IgG, and then with the substrate. In panel A, H and L are mouse IgG heavy and light chains derived from FU-MK-1 used for immunoprecipitation.

lane C). These results indicate that the FU-MK-1 epitope is localized in the GA733-2 protein.

As shown in Fig. 4B, FU-MK-1 detected all three other recombinant proteins: two bands for Fr. 1 with molecular masses of 33 and 36 kDa (lane 2); one for Fr. 2 with a molecular mass of 23 kDa (lane 3); and two for Fr. 3 with a molecular mass of 17.5 and 18 kDa (lane 4). When COS-1 transfectant cells were treated with tunicamycin, the three recombinant proteins all yielded a single band with molecular masses of 31 and 20, and 15 kDa, respectively (Fig. 4B, lanes 6–8). These results suggest that the FU-MK-1 epitope exists on the peptide consisting of amino acids 24–139 of the GA733-2 protein, considering that the deduced amino acid sequence contains a potential signal sequence with the signal peptidase site located at Ala²³-Gln²⁴.^{9, 10, 12)}

Characterization of antigenic determinants by immunoblotting It has been reported that the GA733-2 antigen might undergo partial spontaneous proteolytic cleavage in some carcinoma cell lines.³¹⁾ The cleavage leads to the formation of 2 polypeptide chains of 34 and 6 kDa, respectively, which seem to remain linked by disulfide bonds.³¹⁾ In order to characterize this cleavage and the antigenic determinant localization in the MK-1 antigen, the FU-MK-1 immunoprecipitates of MKN-45 cells lysed under hypotonic condition were examined by immunoblotting analysis using Ch FU-MK-1 after SDS-PAGE in 15% gels. In the Amidoblack staining, the MK-1 antigen was predominantly cleaved into 2 characteristic fragments of 34 and 6 kDa after treatment under hypotonic conditions, although two native forms with high molecular masses of 40 and 42 kDa still remained (Fig. 5A, lanes 1 and 2). The separation into 2 polypeptide chains was detectable only after reduction of the immunoprecipitates, indicating that the 2 chains are linked by disulfide bonds (Fig. 5A, lanes 2 and 4). In the immunostaining, FU-MK-1 antibody recognized the 6-kDa fragment as well as two native forms (40 and 42 kDa), but did not recognize the 34-kDa form (Fig. 5B, lanes 1 and 2).

DISCUSSION

In the present study, we tried to identify the antigen defined by the anti-gastric adenocarcinoma MAb FU-MK-1. Flow cytometric analysis of various human tumor cell lines showed that FU-MK-1 reacts with most carcinoma cell lines. This result is similar to that obtained with MAb GA733.¹¹⁾ However, the reason why the frequency of MK-1-positive cells is high in colonic carcinomas and is low or almost zero in urological carcinomas is unclear at present. A possible explanation is that the expression of MK-1 might be dependent on the degree of malignancy of each cell line. Immunoprecipitation studies of different tumor cell lines revealed that the FU-MK-1 target molecule is a

glycoprotein with two forms, 40 kDa (major form) and 42 kDa (minor form), and with a molecular mass of 35 kDa following treatment with tunicamycin. Although the amino acid sequences of these two forms (40 and 42 kDa) could not be identified, in addition to the 40- and 42-kDa forms, we also detected a 34-kDa form as a major component after hypotonic treatment of living cells and succeeded in determining 17 contiguous amino acids by NH₂-terminal sequencing. The sequence of this smaller 34-kDa form starts with residue 81 (Arg) of the amino-acid sequence derived from the cloned GA733-2 cDNA.¹²⁾ Taken together with the immunohistological findings,¹⁾ the data presented in this study strongly suggested that FU-MK-1 might recognize the tumor-associated antigen GA733-2. This antigen, which is expressed in both normal and cancerous colon epithelial cells, has been independently cloned by several groups, and referred to as KSA by Strnad *et al.*,⁹⁾ KS1/4 antigen by Perez and Walker,¹⁰⁾ and EGP-2 by Simon *et al.*¹⁴⁾ Based on hydrophobicity analysis of the predicted antigen sequence, GA733-2 resembles a type I membrane protein. An amino-terminal 23-residue signal peptide is followed by a 242-residue extracellular domain containing 12 cysteine residues and 3 potential N-glycosylation sites, a 23-residue transmembrane domain, and a highly charged 26-residue intracellular anchor.^{9, 12)} (Fig. 4A). Final confirmation of the specificity of FU-MK-1 came from the study of COS-1 cells transfected with a cDNA coding for the GA733-2 antigen. FU-MK-1 reactivity was demonstrated by immunoprecipitation of the expressed GA733-2 cDNA in the COS-1 expression system, thus providing data which substantiate the notion that the FU-MK-1 and GA733-2 antigens might be one and the same molecular entity.

Several antibodies have been generated directed against GA733-2,¹⁷⁾ also known as the 17-1A antigen,⁵⁾ the Trop-1 antigen,⁶⁾ KSA,⁹⁾ EGP-2,¹³⁾ the C215 antigen,¹⁵⁾ etc. Based on chemical analysis of the epitopes and cross-competition assays among them, de Leij *et al.*¹⁷⁾ suggested that several different epitopes exist on the GA733-2 molecule. One epitope is defined by only one antibody MM104.^{31, 32)} Antibodies MJ37 and MP75^{31, 32)} are directed against the same epitope, and RS11-51,³³⁾ GA733,¹¹⁾ and 17-1A⁴⁾ are all directed against another epitope. Antibodies MH99,^{31, 34)} KS1/4,⁸⁾ AUAI,³⁵⁾ MM121,^{31, 32)} and Trop-1⁶⁾ block the binding of both groups of antibodies, indicating that the antigenic structure recognized by these antibodies partially overlaps both of the epitopes detected by the other groups of anti-GA733-2 antibodies. Recently, we have cloned the variable region genes of FU-MK-1²⁾ and compared them with those of MAbs GA733 and CO17-1A.³⁶⁾ Relatively low similarities between them were found, suggesting that the epitope recognized by FU-MK-1 might be different from those recognized by the others.

The exact relationship of FU-MK-1 to the other MABs against GA733-2, however, remains to be clarified.

Since only a few authors have described the specificity of their MABs in terms of protein structure,¹⁶⁾ we constructed three cDNAs encoding amino acids 1–265, 1–201, and 1–139 of the GA733-2 protein, respectively (Fig. 4A). The reactivity with the three recombinant GA733-2 proteins revealed that the epitope for FU-MK-1 is present on the distal half of the extracellular domain, because no repeated sequences were found in the structure of the GA733-2 protein.¹²⁾ Furthermore, the hypotonic treatment of living tumor cells yields two specifically sized breakdown products (34 and 6 kDa) of the MK-1 molecule, which remain linked by disulfide bonds (Fig. 5). Comparison of amino acid sequencing data with the predicted amino acid sequence of the GA733-2 reveals that the 34-kDa form most likely results from a dibasic cleavage between Arg⁸⁰-Arg⁸¹.^{10, 16)} The data presented here suggest that this may be a non-specific proteolytic cleavage occurring during hypotonic treatment of living cells. In immunoblotting analysis, FU-MK-1 bound to the 6-kDa fragment and not to the 34-kDa one. This observation indicated that FU-MK-1 recognizes an epitope on the distal 6-kDa fragment (amino acids 24–80) of the extracellular

domain of the GA733-2 molecule, considered a potential signal peptide.^{9, 10, 12)}

The primary aim of the present investigation was to identify the MK-1 antigen. Our findings indicate a close relationship between the MK-1 antigen and the GA733-2 antigen, which has recently been reported to be effective as a target in clinical trials with monoclonal antibodies. The favorable results reported in colon adenocarcinoma patients treated with the MAb CO17-1A^{24, 25)} suggest that FU-MK-1 may have potential as an antibody for use in tumor imaging or immunotherapy of various carcinomas.

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