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Development of monoclonal antibodies to human microsomal epoxide hydrolase and analysis of "preneoplastic antigen"-like molecules

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ABSTRACT

Microsomal epoxide hydrolase (mEH) is a drug metabolizing enzyme which resides on the endoplasmic reticulum (ER) membrane and catalyzes the hydration of reactive epoxide intermediates that are formed by cytochrome P450s. mEH is also thought to have a role in bile acid transport on the plasma membrane of hepatocytes. It is speculated that efficient execution of such multiple functions is secured by its orientation and association with cytochrome P450 enzymes on the ER membrane and formation of a multiple transport system on the plasma membrane. In certain disease status, mEH loses its association with the membrane and can be detected as distinct antigens in the cytosol of preneoplastic foci of liver (preneoplastic antigen), in the serum in association with hepatitis C virus infection (AN antigen), or in some brain tumors. To analyze the antigenic structures of mEH in physiological and pathological conditions, we developed monoclonal antibodies against different portions of mEH. Five different kinds of antibodies were obtained: three, anti-N-terminal portions; one anti-C-terminal; and one, anti-conformational epitope. By combining these antibodies, we developed antigen detection methods which are specific to either the membrane-bound form or the linearized form of mEH. These methods detected mEH in the culture medium released from a hepatocellular carcinoma cell line and a glioblastoma cell line, which was found to be a multimolecular complex with a unique antigenic structure different from that of the membrane-bound form of mEH. These antibodies and antigen detection methods may be useful to study pathological changes of mEH in various human diseases.

Introduction

The microsomal epoxide hydrolase (mEH) is a drug-metabolizing enzyme that converts epoxides to diols and plays an important role in the metabolism of some mutagenic and carcinogenic epoxides (Newman et al., 2005). It is mainly expressed on the ER membrane in the liver (Newman et al., 2005) and constitutes about 2% of microsomal proteins (Gill et al., 1982). It was reported that mEH associates with various cytochrome *P450*s (Holder et al., 1974; Ishii et al., 2005), and functional cooperation between those enzymes is suggested (Taura et al., 2002). It is also expressed on the surface of hepatocytes (Zhu et al., 1999) and may act as a sodium-dependent bile acid transporter (von Dippe et al., 2003). In humans, mEH is the product of single locus (EPHX1) on chromosome 1. Several single nucleotide polymorphism sequences were found in association with the onset of several diseases and cancers (McGlynn et al., 1995; Park et al., 2005; Smith and Harrison, 1997; Sonzogni et al., 2002). Association of the mEH with cancers and diseases has been further suggested by the following observations. The mEH was identified as the brain tumor antigen in some glioblastoma cell lines (Kessler et al., 2000). Although mEH is

Abbreviations: mEH, microsomal epoxide hydrolase; ER, endoplasmic reticulum; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; CsCl, cesium chloride; PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactoside; GST, glutathione S-transferase; TMB, 3, 37, 5, 57-tetramethylbenzidone; BCIP, 5-bromo-4-chloro-3-indoyl-phosphate; NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HRP, horseradish peroxidase; FCS, fetal calf serum; CBA, competitive antibody binding assay; S-mEH, solubilized form of mEH; M-mEH, membrane-bound form of mEH; L-mEH, linearized form of mEH; RIA, radioimmunoassay; PNA, preneoplastic antigen; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; OD, optical density.

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tightly associated with membranes in normal cells, the mEH is sometimes detected in the cytosol of neoplastic human livers and released into the blood (Gill et al., 1983). The appearance of mEH in the blood is also associated with other types of liver disease (Hammock et al., 1984). In hepatitis C infection, we have shown that the hepatitis C-associated antigen (AN antigen) appears in the early phase of the viral infection (Akatsuka et al., 1986b), which is followed by the development of the antibody in the acute phase of hepatitis (Akatsuka et al., 1986a). In a recent study, we have shown that the AN antigen is mainly composed of mEH (Akatsuka et al., 2007). These lines of evidence suggest that mEH loses the association with membranes in some disease processes which are accompanied by the changes of its structure. To enable us to analyze the changes of mEH status in situ, we developed monoclonal antibodies against different parts of mEH molecule and antigen detection methods which can quantitate differentially the membrane-bound form and soluble form of mEH.

Materials and methods

Cell lines. Sf9 was a gift from Dr. Stephen M. Feinstone (CBER, FDA) and cultured in Sf900II SFM (Invitrogen, Carlsbad, CA) at 27 °C. The hepatocellular carcinoma (HCC) cell lines, Huh-1 (Huh et al., 1982) and Huh-7 (Nakabayashi et al., 1982) were obtained from the Japanese Cancer Research Resources Bank. Huh-1 was cultured in RPMI1640 with 10% FCS; Huh-7 was cultured in RPMI1640 with 2% FCS and 30 nmol/l Na_2SeO_3. THLE-5b, a normal liver cell line which was immortalized by transfecting with the plasmid containing SV40 T antigen (Lechner et al., 1991), was a gift from Dr. Curt C. Harris (NCI, NIH) and cultured in RPMI1640 with 5% FCS. Human fibroblast cell line M1 (Royer-Pokora et al., 1984) was a gift from Dr. William E. Biddison (NIAID, NIH). Glioblastoma cell lines U87MG (Ishii et al., 1999) and LN-Z308 (Albertoni et al., 1998) were gifts from Dr. Ryo Nishikawa (Saitama Medical Univ., Japan) and LN-71 (Ishii et al., 1999) was a gift from Dr. Erwin G. Van Meir (Emory Univ.). These four cell lines were cultured in DMEM with 10% FCS. Myeloma cell line NS-1 was a gift from Dr. Mineo Arita (National Institute of Infectious Diseases, Japan) and cultured in RPMI1640 with 15% FCS.

Purified mEH antigens. Expression of human mEH in a recombinant baculovirus system (Morisseau et al., 2001) and the purification procedure of the solubilized form of mEH (Akatsuka et al., 2007) have been described. Briefly, the infected cells were solubilized with Triton X-100 and subjected to Q-Sepharose column chromatography. Antigen-positive fractions were pooled and stored in a buffer containing 0.05% Triton X-100. A part of this preparation (the solubilized form of mEH, S-mEH) was used for ELISA screening of hybridoma cultures, and the rest was submitted for preparative SDS-PAGE to obtain the linearized form of mEH (L-mEH) which was used for immunization of mice, competitive antibody binding assays, and as the standard for antigen detection assays. The preparative SDS-PAGE was performed by applying 1.0 mg of the S-mEH to Model 491 PrepCell (Bio-Rad, Hercules, CA) consisting of a 40 ml polyacrylamide (10%) gel and a 6 ml stacking gel. During electrophoresis at 12 W, proteins were eluted with running buffer with a flow rate of 0.5 ml/min, and 3.5 ml fractions were collected. Each fraction was tested by dot blot assay on an Immobilon-P membrane (Millipore, Bedford, MA) using rabbit anti-mEH antibody (Akatsuka et al., 2007), and then, aliquots of antigen-positive fractions were subjected to a 10% minigel (Mini-Protean; Bio-Rad, Hercules, CA) for silver staining. Factions with a homogenous 47 kDa band were pooled, applied to a 0.4 ml Extracti-Gel column (Pierce, Rockford, IL), and after elution with PBS containing 0.05% Triton X-100, concentrated with Centriplus YM-10 (Millipore, Bedford, MA). Purification of the membrane-bound form of mEH (M-mEH) has been described (Akatsuka et al., 2007). Briefly, a homogenate of recombinant baculovirus-infected Sf9 cells was first passed through a gel-filtration column (Sephacryl S-300, Amersham, Uppsala, Sweden). The void volume fraction was then subjected to ultracentrifugation in a sucrose-gradient followed by a second ultracentrifugation in a CsCl gradient. Each fraction was tested by an antibody sandwich ELISA using horseradish peroxidase (HRP)-labeled anti-AN antigen monoclonal antibody 1F12, and positive fractions were pooled and concentrated with Centriplus YM-100 (Millipore, Bedford, MA). Protein concentration was measured by micro BCA assay (Pierce, Rockford, IL).

GST-mEHs. A full-length and eight truncated human mEH cDNAs (Fig. 1) were amplified using the mEH cDNA (Akatsuka et al., 2007) by PCR with KOD-Plus polymerase (Toyobo, Tokyo, Japan) and primers shown in Table 1. The PCR products were digested with *Bam*HI and *Sma*I, and ligated into the same cloning sites of pGEX-2T (GE Healthcare, Uppsala, Sweden). Competent BL21 cells of *E. coli* were transformed with the recombinants, and after IPTG induction (0.1 mM, 5 h), 1 ml culture of the cells was extracted with 300 μl of SDS sample buffer and used as the ELISA antigen.

SDS-PAGE and Western blotting. For Coomassie blue staining and immunoblotting of GST-mEH produced by *E. coli*, 13 and 5 μl of cell suspensions were extracted with SDS sample buffer and applied onto a 10% minigel (Mini-Protean; Bio-Rad), respectively. After separation and transfer to an Immobilon-P membrane (Millipore, Bedford, MA), the antigen was detected with goat anti-GST (Amersham, Buckinghamshire, UK) (1:1000) followed by HRP-labeled rabbit anti-goat IgG (KPL, Gaithersburg, MA) and TMB substrate (KPL), or with mouse anti-mEH obtained in this study (1:500) followed by alkaline phosphatase-labeled goat anti-mouse IgG (KPL) and BCIP/NBT substrate (KPL), respectively. For the immunoblotting of mEH-expressing cell lines, 1×10^5 cells were extracted in 25 μl of SDS sample buffer and applied onto a 10% minigel. The transferred antigen was detected with the culture supernatant of anti-mEH hybridoma culture (1:10) followed by alkaline phosphatase-labeled goat anti-mouse IgG (KPL) and BCIP/NBT substrate (KPL).

ELISA for antibody detection. Each well of 96-well ELISA plates (Costar, Acton, MA) received 50 μl of one of the following antigen solutions diluted in PBS: M-mEH, S-mEH, and L-mEH, 0.4 μg/ml; GST-mEH, (1:250) dilution; and the mEH peptide aa 54–71, 0.5 μg/ml. After incubation at 4 °C overnight, the well was washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 200 μl of PBS containing 5% BSA (blocking solution) for 1 h at 37 °C. After washing, 50 μl of culture supernatants, ascitic fluids, or sera diluted in PBS containing 1% BSA (dilution buffer) was added and incubated at 37 °C for 1 h. Each well was washed three times and received 50 μl of HRP-conjugated anti-mouse IgG or anti-rabbit IgG (H- and L-chain specific) (KPL, Gaithersburg, MA) which was diluted 1:1000 (0.5 μg/ml) with PBS containing 10% FCS. After incubation at 37 °C for 1 h and washing, the color was developed by adding 200 μl of *o*-phenylenediamine dihydrochloride substrate (SIGMA, St. Louis, MO), and the reaction was stopped by adding 50 μl of 6 N H_2SO_4. The plates were measured at an optical density of 492 nm. We previously described the aa 54–71 mEH peptide, the rabbit antibody against this peptide (Maekawa et al., 2003), and the rabbit anti-mEH antibody (Akatsuka, et al., 2007).

Monoclonal antibodies. Two female BALB/c mice (age, 6 weeks) were purchased from Tokyo Laboratory Animal Science Co. Ltd. (Tokyo, Japan). They were injected s.c. with 2 μg of L-mEH four times (first with Freund's complete adjuvant, second with incomplete adjuvant, third and fourth immunizations without adjuvant) at one- to two-month intervals. Two months thereafter, they were boosted i.p. with 40 μg of L-mEH, and after another 3 days, their spleen cells were harvested. Hybridization of spleen cells with myeloma cells NS-1 was performed as described (Akatsuka et al., 1986b), and culture supernatants were tested for anti-mEH activity by ELISA using S-mEH as the antigen. Antibody-secreting cells were cloned three times or more by

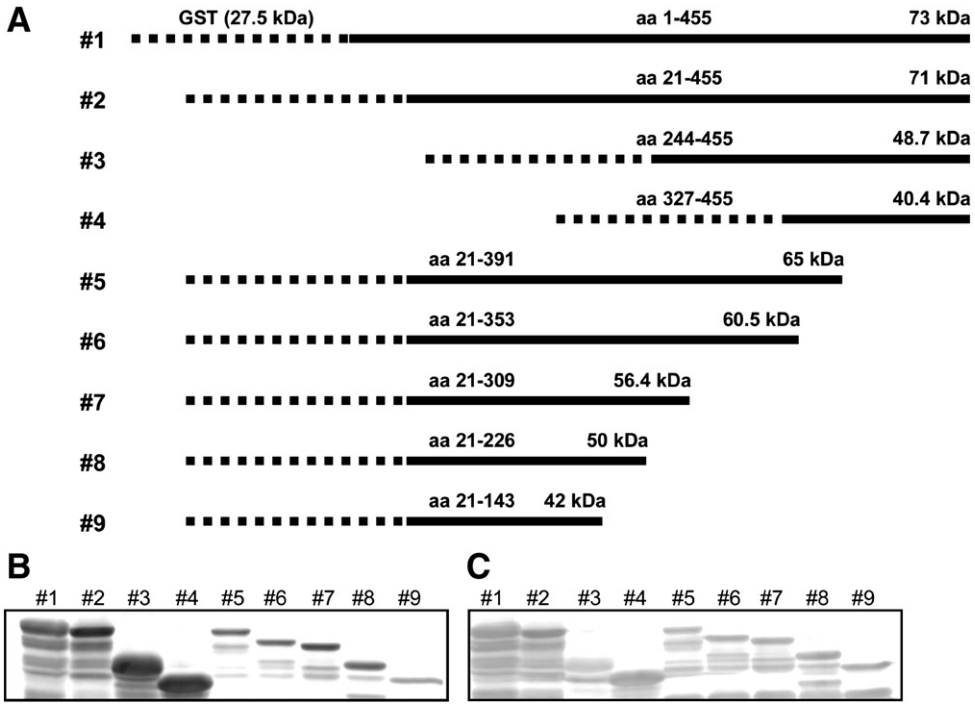

Fig. 1. Full-length and truncated mEH expressed as GST fusion proteins in *E. coli*. A, schematic representation of nine kinds of GST-mEH (F-mEH) used for epitope mapping. A predicted size (shown by kDa) of a GST-mEH from each construct is shown on the right. Expressed antigens were detected by Western blotting with goat anti-GST (B) and mouse anti-mEH (C) polyclonal antibodies and confirmed to have the expected molecular sizes.

limiting dilution with the culture medium containing 10% BM-Conditioned H1 (Roche, Mannheim, Germany). Ascitic fluids were obtained by injecting hybridomas i.p. into pristane (SIGMA)-primed BALB/c nude mice (Clea Japan, Tokyo, Japan) ($1–5\times10^6$ cells/mouse). Antibody isotype was determined with mouse monoclonal antibody isotyping kit (GE Healthcare) following the manufacturer's instructions.

Competitive antibody binding assay. Competitive antibody binding assay (CBA) was performed following the procedure described elsewhere (Stone and Nowinski, 1980). A flat-bottomed eight well strip plate (Costar) was used as the solid phase and 50 μl of L-mEH diluted (0.4 μg/ml) in PBS was added to each well and incubated overnight at 4 °C. For the assays between the type V and the anti-AN antibody, M-mEH was coated instead of L-mEH to each well. After blocking with 200 μl of blocking solution for 1 h at 37 °C and washing, 50 μl of unlabeled IgG (5-fold dilutions in dilution buffer starting from 5 μg/ml) was incubated in each well at 37 °C for 1 h. Then, 2×10^5 cpm of ^{125}I-labeled IgG in 50 μl of dilution buffer was added and incubated at 37 °C for 1 h. Each well was separated after washing, and the bound radioactivity was counted by a gammacounter. IgG was purified from ascitic fluid using Protein G Sepharose (GE Healthcare) following the manufacturer's instructions, and labeling with ^{125}I was performed by the chloramine-T method (Mcconahey and Dixon, 1966).

ELISA and RIA for Ag detection. IgG was labeled with ^{125}I as described above or with HRP using Peroxidase Labeling Kit (Dojindo, Kumamoto, Japan). Antibody sandwich RIA and ELISA were performed using flat-bottomed eight well strip plates (Costar) and 96-well ELISA plates (Costar), respectively. Each well adsorbed 50 μl of unlabeled IgG (5 μg/ml in PBS; 1 h at 37 °C), and after washing with PBS-T and blocking, received 50 μl of antigen solution in dilution buffer. After incubation and washing, 1×10^5 cpm of ^{125}I-labeled IgG or HRP-labeled IgG in 50 μl of dilution buffer was added, and after another incubation and washing, the bound radioactivity was counted by a gammacounter or the color was developed by adding *o*-phenylenediamine dihydrochloride substrate as described above for CBA and Ag binding ELISA. Culture supernatants used for mEH antigen detection were prepared as follows: the cells were seeded in 10-cm dishes, and after 4 days incubation (~80% confluency), the supernatant was collected, and after centrifugation at 30,000×*g* for 10 min, filtrated through a 0.45 μM filter and stored at −80 °C until use. Aliquots of the culture supernatant were concentrated about 10-fold by ultrafiltration through

Table 1
Primers for the amplification and expression of mEH fragments.

Primer	Sequence[a]	Description
1	5′-CGGGATCCATGTGGCTAGAAATCCTCCTCAC-3′	Sense primer for #1
2	5′-CGGGATCCCGGGACAAAGAGGAAACTTTGCC-3′	Sense primer for #2, #5–#9
3	5′-CGGGATCCAAAGGCCTGCACTTGAACATGGC-3′	Sense primer for #3
4	5′-CGGGATCCGAGAAGTTTTCCACCTGGACC-3′	Sense primer for #4
5	5′-CGGGATCCTTGCCGCTCCAGCACCGACAGG-3′	Antisense primer for #1
6	5′-TCCCCCGGGTTGCCGCTCCAGCACCGACAGG-3′	Antisense primer for #2–#4
7	5′-TCCCCCGGGCTTCATCCGCTCACGCTTCTG-3′	Antisense primer for #5
8	5′-TCCCCCGGGTCGTCCAGGGAGAACTTCC-3′	Antisense primer for #6
9	5′-TCCCCCGGGGGTGTCAGGCTTGGTGCACTG-3′	Antisense primer for #7
10	5′-TCCCCCGGGGTCCCCTCCTTGAATGTAGAA-3′	Antisense primer for #8
11	5′-TCCCCCGGGGGGCTTCGGGGTATGGCCTGC-3′	Antisense primer for #9

[a] The underlined sequences GGATCC and CCCGGG represent recognition sequences of *Bam*HI and *Sma*I, respectively.

Table 2
Epitope mapping of anti-mEH monoclonal antibodies by ELISA. Mean OD values of duplicate ELISA are indicated, and positive results are highlighted in gray. The cut-off was determined as the mean ± 5 standard deviations of NS-1 culture supernatants (0.113) and that of the naïve mouse sera (0.202), for hybridoma cultures and the immune mouse sera, respectively.

	F-mEH									
Antibody	1	2	3	4	5	6	7	8	9	pGEX
2D8	2.790	2.637	0.059	0.067	2.721	2.833	2.695	2.686	2.757	0.055
5D8	2.551	2.365	0.092	0.063	1.824	1.972	2.055	2.051	2.001	0.053
8F11	2.519	2.330	0.090	0.050	1.819	1.663	2.065	2.043	1.852	0.053
K4F8	2.473	2.354	0.105	0.096	1.785	1.931	1.987	1.938	1.752	0.062
K2B7	2.527	2.395	0.086	0.082	0.913	0.930	1.341	1.475	0.637	0.059
6E3	2.120	2.029	2.779	2.770	0.214	2.022	0.064	0.068	0.075	0.032
2G2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
7B11	0.007	-0.016	-0.027	0.031	-0.026	-0.101	-0.023	-0.048	-0.041	0.001
NS-1	0.061	0.070	0.046	0.077	0.062	0.064	0.057	0.052	0.045	0.045
Immune	1.657	1.417	2.199	2.092	1.199	1.204	1.424	1.288	1.185	0.117
Naïve	0.060	0.046	-0.015	0.048	0.064	-0.009	0.058	0.051	0.087	0.038

a 10 kDa cut-off membrane (Centriplus YM-10, Millipore) and half of each concentrate was further subjected to ultrafiltration through a100 kDa cut-off membrane (Centricon YM-100, Millipore) and the flow-through fractions were collected.

Analysis of mEH activity in culture media and inhibitory activity of monoclonal antibodies. Concentrated culture media were dissolved 10 fold in Tris/HCl buffer (0.1 M pH 9.0) containing 0.1 mg/ml BSA and the activities were measured with [^{3}H]-*cis*-stilbene oxide after incubation for 15 min at 30 °C (Morisseau et al., 2001). Purified human mEH (S-mEH) diluted 100-fold was used as positive control, and protein content was measured with BCA assay (Pierce, Rockford, IL) using BSA as standard. Detection limit in this condition was around 0.3 nmol min^{-1} ml^{-1}. Monoclonal antibodies were diluted 10^7 fold in the same buffer as above and incubated with S-mEH. mEH inhibitor, 2-nonylthio-propiamide (NTPA) was used at 100 μM as the positive control.

Results

Preparation of mEH antigens for immunization and antibody detection

We developed monoclonal antibodies which recognize different portions of human mEH. The solubilized form of mEH (S-mEH) was purified as described previously (Akatsuka et al., 2007) and used for the screening of hybridomas. A part of S-mEH was submitted for preparative SDS-PAGE to obtain the linearized form of mEH (L-mEH) and used for immunization of mice, because we wished to obtain antibodies against the linear epitopes of mEH (see Discussion). For epitope mapping of monoclonal antibodies, we expressed nine mEH fragments (F-mEH) with truncations at the N-terminus or the C-terminus as GST-fusion proteins in *E. coli* (Fig. 1A). SDS-PAGE followed by Coomassie Blue staining (data not shown) or immunoblotting with an anti-GST antibody (Fig. 1B) and anti-mEH antibody (Fig. 1C) revealed that each mEH fragment with the predicted size was successfully expressed in *E. coli*. ELISA testing using the F-mEH 1 to 4 as the antigens showed that the immunized mice developed antibodies against F-mEH 1 and 2 but not 3 or 4 after the first and the second immunizations. Antibodies which reacted with all the four antigens appeared only after the third immunization, which suggested that the N-terminus of mEH had higher immunogenicity than the C-terminus (data not shown).

Development of monoclonal antibodies against mEH

After we confirmed that the two immunized mice developed antibodies to F-mEH 1 to 4, we established hybridomas. In two separate fusions, sixty-five colonies were found to produce antibodies against the S-mEH, among which 23 antibodies reacted only with F-mEH 1 and 2, 16 reacted with F-mEH 1 to 4, and 26 reacted only with the S-mEH. Eight hybridomas were subjected to limiting dilution three times or more, and tested by ELISA using all of the GST-mEH antigens (F-mEH 1 to 9). The results shown in Table 2 suggest that the five antibodies (2D8, 5D8, 8F11, K4F8, and K2B7) recognize the N-terminus (aa 21–143, F-mEH 9), and 6E3 recognizes the C-terminus (aa 327–353, F-mEH 4). Antibodies 2G2 and 7B11 reacted with the S-mEH but not with any of the F-mEH 1 to 9, therefore, they seemed to recognize the conformational epitope which was lost during the preparation of the F-mEHs by SDS treatment (Fig. 2). This speculation was substantiated by the ELISA in which the antibodies were tested against the three antigens: the S-mEH, the L-mEH, and the membrane-bound form of mEH (M-mEH). The antibodies 2G2, 7B11, and the anti-AN antigen monoclonal antibodies 1H9 and 1F12, which recognize the three-dimensional structure of mEH (Akatsuka et al., 2007), reacted with M-mEH and S-mEH but not with L-mEH.

The six antibodies which reacted with one or more of the F-mEH 1 to 9 seemed to recognize linear structures of mEH, and were tested for their reactivity in Western blotting using three kinds of mEH-expressing cell lines: Sf9 cells infected with a recombinant baculovirus (Fig. 3A), THLE-5b, a normal human liver cell line which was immortalized by transfecting with the plasmid containing SV40 T-antigen (Lechner et al., 1991) (data not shown), and a hepatocellular carcinoma

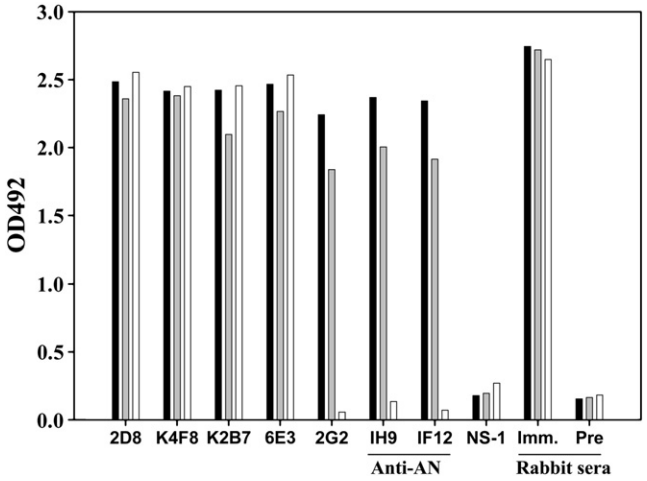

Fig. 2. ELISA test for the measurement of antibody reactivity to M-mEH (black bars), S-mEH (gray bars), and L-mEH (white bars). Ascitic fluids of hybridomas (1:1000 dilution) and the rabbit anti-mEH antiserum (1:200 dilution) were tested. The ascitic fluid of NS-1 cells and the preimmune rabbit serum were used as the negative controls.

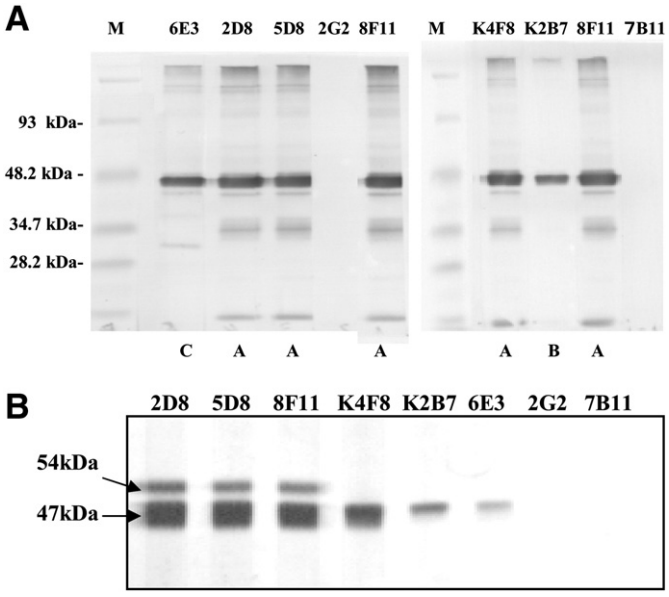

Fig. 3. Reactivity of anti-mEH monoclonal antibodies against the immunoblots of mEH-expressing cell lines. 10% SDS gel was loaded with molecular weight marker (M), mEH-expressing Sf9 cell extract (A), and Huh-1 cell extract (B), and underwent Western blotting. The immunoblot was detected with anti-mEH antibodies followed by alkaline phosphatase-labeled second antibody. The patterns (A–C) of the bands on the blot of Sf9 cell extract (A) are shown at the bottom of the figure.

(HCC) cell line, Huh-1 (Huh et al., 1982) (Fig. 3B). When extracts of each of the three cell lines were blotted, all of the six antibodies showed 47 kDa bands, the size of which corresponded to the whole mEH molecule. In the blotting of mEH-expressing Sf9 cells (Fig. 3A), additional smaller bands, which may be degradation products of mEH, could be seen, and the pattern of the bands could be divided into three types: type A for 2D8, 5D8, 8F11 and K4F8; type B for K2B7; and type C for 6E3. Based on these patterns of bands, the five antibodies which recognize the N-terminus (aa 21–143) could be separated into the two groups: type A group (2D8, 5D8, 8F11 and K4F8) and type B group (K2B7) (Table 3). In the blotting of the hepatocyte-derived cell lines THLE-5b and Huh-1, a higher band of 54 kDa could be seen with the three out of four antibodies in the type A group (2D8, 5D8 and 8F11), but not with K4F8 in the same type A group or other four antibodies (Fig. 3B). Therefore, the four antibodies in the type A group could be further divided into the two groups: 2D8, 5D8 and 8F11 (type A1), which cross-react with a molecule of 54 kDa protein, and K4F8 (type A2), which only recognizes a 47 kDa band (Table 3). Therefore, the eight antibodies could be divided into the five types (type I to V) depending on their epitope specificities (Table 3).

The epitope specificity of the antibodies was further tested by competitive antibody binding assay (CBA) in which a constant amount of ^{125}I-labeled antibody was allowed to compete with increasing concentrations of the unlabeled homologous or heterologous antibodies for binding to L-mEH. As an unlabeled antibody, rabbit antiserum which was raised against a peptide (aa 54–71) (Maekawa et al., 2003) was also included. As shown in Table 4, binding of labeled 5D8 was inhibited by 2D8 as well as by homologous unlabeled 5D8, but not by other types of antibodies, which indicates the two kinds of type I antibodies (2D8 and 5D8) recognize the same or closely adjacent epitopes. Binding of K4F8 (type II) was greatly inhibited by the two type I antibodies as well as homologous K4F8. Although inhibition of binding of labeled 5D8 by unlabeled K4F8 was relatively low, type I and type II antibodies seemed to recognize distinct but adjacent epitopes on mEH. Binding of labeled K2B7 was almost completely inhibited by the rabbit anti-peptide antibody as well as by homologous unlabeled K2B7, which indicated that the epitope of type III overlaps with the region of aa 54–71. Binding of labeled 6E3 was inhibited only by homologous unlabeled 6E3. CBA was also performed for the antibodies which recognize conformational epitopes using M-mEH as the antigen. Binding of type V antibody 2G2 was found to be inhibited not only by homologous 2G2 but also by 1H9 which had been developed against the AN antigen purified from the liver of a patient with hepatitis C (Akatsuka et al., 1986b) and later proved to be highly selective for a conformational epitope on the membrane-bound form of mEH (Akatsuka et al., 2007). To confirm the possibility that the epitope of K2B7 (type III) overlaps with aa 54–71, we tested the antibodies by ELISA with the peptide aa 54–71 coated to the plates, and found that only K2B7 and the rabbit antiserum revealed significant reactivity with the peptide (Fig. 4). Taken together, the epitope selectivity of the five types of the antibody can be summarized as shown in Table 5. These five types of antibodies were tested if they inhibit mEH catalytic activity, but any of the antibodies did not reveal significant inhibition against purified recombinant human mEH (S-mEH) (less than 13.3%, data not shown).

Development of mEH antigen detection systems

Previously, we have detected a hepatitis C-related antigen (AN-antigen) in sera from patients with hepatitis C (Akatsuka et al., 1986b) and chimpanzees experimentally infected with hepatitis C virus (HCV) (Akatsuka et al., 1986a) by radioimmunoassay (RIA) using anti-AN-antigen monoclonal antibody, 1F12. Recently, we have shown that most of the antigenicity of AN-antigen consists of mEH and anti-AN-antigen monoclonal antibodies recognize conformational epitopes of mEH (Akatsuka et al., 2007). The antigen was somewhat similar to preneoplastic antigen (PNA) which had been described as an antigen in preneoplastic foci in livers that is released into the blood (Okita and Farber, 1975), and later found to be immunologically identical to mEH (Levin et al., 1978). The mEH has been also reported to reveal a somewhat anomalous antigenicity in some glioblastoma cell lines (Kessler et al., 2000). To assess the similarity between PNA, AN-

Table 3
Classification of anti-mEH monoclonal antibodies. Eight monoclonal antibodies were grouped into five groups (Type I to V) depending on the reactivity with F-mEH 1 to 4 and S-mEH by ELISA, and the band sizes and patterns they produced in Western blotting. Their H-chain and L-chain isotypes are also shown.

Clone	Isotype	L-chain	ELISA					Western blotting			
			F-mEH								
			1	2	3	4	S-mEH	Sf9	THLE-5b	Huh-1	Type
2D8	G2a	κ	+	+	−	−	+	A1	54 K, 47 K	54 K, 47 K	I
5D8	G1	κ	+	+	−	−	+	A1	54 K, 47 K	54 K, 47 K	I
8F11	G1	κ	+	+	−	−	+	A1	54 K, 47 K	54 K, 47 K	I
K4F8	G1	κ	+	+	−	−	+	A2	47 K	47 K	II
K2B7	G1	κ	+	+	−	−	+	B	47 K	47 K	III
6E3	G1	κ	+	+	+	+	+	C	47 K	47 K	IV
2G2	G1	κ	−	−	−	−	+	−	−	−	V
7B11	M	κ	−	−	−	−	+	−	−	−	V

Table 4
Competitive binding assay. Six kinds of antibodies were labeled with ^{125}I, and 2×10^5 cpm of each antibody was incubated with increasing concentrations of the unlabeled homologous or heterologous antibodies in the wells coated with mEH. As the antigen, L-mEH was used for the antibodies from type I to type IV and the rabbit anti-peptide aa54–71 antibody. M-mEH was used for the type V and anti-AN antibodies. % inhibition values at 5 μg/ml are shown, and those above 80% are highlighted in gray.

^{125}I-antibody		Unlabeled antibody							
		Type I		Type II	Type III	Type IV	Type V	Anti-AN	Rabbit Ab
Type	Clone	2D8	5D8	K4F8	K2B7	6E3	2G2	1H9	aa54-71
Type I	5D8	96.1	100.0	62.6	30.9	38.0	1.4	1.4	4.2
Type II	K4F8	93.9	94.7	100.0	16.9	10.5	15.5	15.5	25.9
Type III	K2B7	28.4	54.2	72.0	100.0	39.7	0.0	0.0	99.3
Type IV	6E3	30.0	26.2	43.4	44.0	100.0	43.0	43.0	51.6
Type V	2G2	N.D.	17.9	N.D.	N.D.	9.1	100.0	100.0	N.D.
Anti-AN	1H9	N.D.	14.1	N.D.	N.D.	10.7	87.8	100.0	N.D.

N.D.: not determined.

antigen, and the brain tumor antigen, and their differences from the normal mEH bound to the membrane, we developed sensitive mEH antigen detection assay systems using the monoclonal antibodies. Among the five types of antibodies, type I antibodies which cross-react with a 54 kDa protein were excluded, and K4F8 (type II), K2B7 (type III), 6E3 (type IV), and 2G2 (type V) were labeled with ^{125}I or horseradish peroxidase (HRP). Two kinds of the purified mEH obtained from recombinant baculovirus-infected Sf9 cells were used as the antigen standards. The membrane-bound form of mEH was purified by density gradient centrifugation without using detergents (Akatsuka et al., 2007) (M-mEH); the linearized form of mEH (L-mEH) was described above. We tried to detect the antigens by antibody sandwich RIA and ELISA methods using several combinations of unlabeled and labeled antibodies. As shown in Table 6 (titration curves of three kinds of ELISA are shown in Fig. 5), M-mEH was successfully detected by the combinations between type V and homologous or heterologous antibodies; the homologous combination (V–*V) was the most sensitive (Fig. 5A) followed by the combination of unlabeled type IV and labeled type V (IV–*V) (Fig. 5B). L-mEH could be detected by the combination of unlabeled type IV and labeled type II antibodies (IV–*II) (Fig. 5C). Interestingly, M-mEH could not be detected with satisfactory sensitivity by IV–*II (Fig. 5C) or by any other combinations of antibodies excluding type V (Table 6). Importantly, the sandwich assay method based on the combination of IV–*II enables us to detect the mEH antigen in multiple forms only if it is denatured with detergents such as SDS before incubation for the assay.

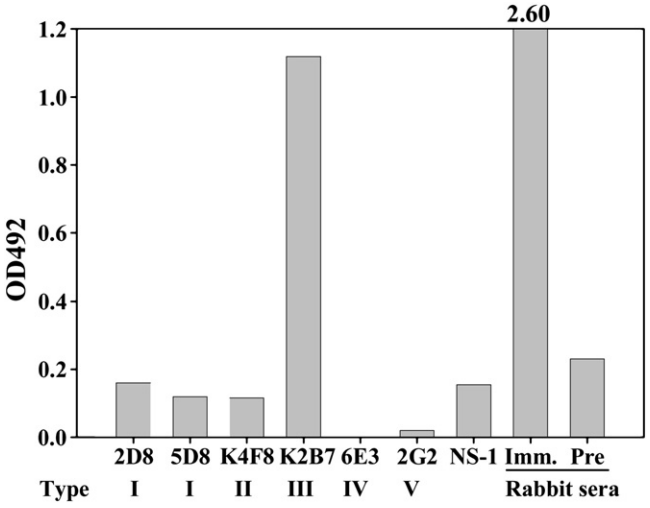

Fig. 4. Reactivities of anti-mEH monoclonal antibodies against the mEH peptide aa 54–71 were tested by ELISA. Ascitic fluids of hybridomas (1:1000 dilution) and the rabbit anti-peptide aa 54–71 antiserum (1:200 dilution) were tested. The ascitic fluid of NS-1 cells and the preimmune rabbit serum were used as the negative controls.

Analyses of antigenic structure of PNA-like mEH antigens

We applied three kinds of RIAs with the antibody combinations, IV–*II, IV–*V, and V–*V to the detection of mEH in culture supernatants of seven cell lines (Table 7). Two lines (THLE-5b and Huh-1) were also used for Western blotting in this study (Table 3). THLE-5b (Lechner et al., 1991) was derived from normal human hepatocytes which were immortalized by transfection of SV40 T antigen gene, and maintain many functions of normal hepatocytes including phase II drug-metabolizing enzymes. Huh-7 (Nakabayashi et al., 1982) is a well-differentiated HCC cell line and secretes a variety of major plasma proteins; e.g., albumin, transferrin alpha-fetoprotein and the acute phase proteins. Huh-1 (Huh et al., 1982) is a HBs antigen-producing undifferentiated HCC line and produces tumors in nude mice. M1 is a cell line derived from human fibroblasts (Royer-Pokora et al., 1984). LN-71 (Ishii et al., 1999), LN-Z308 (Albertoni et al., 1998), and U87MG (Ishii et al., 1999) are human glioblastoma cell lines; LN-71 was described as brain tumor antigen BF7/GF2 positive whereas LN-Z308 as negative (Kessler et al., 2000). All the cell lines were found to express mEH with a 47 kDa band which was detected by the type II antibody K4F8 in Western blotting (data not shown). Significant signals were obtained from the supernatants of Huh-1 and LN-71 by the V–*V method. Those antigens could not be detected by the IV–*V method even though the amounts exhibited by V–*V method (4.26 and 7.83 ng/ml) were higher than the detection limit of IV–*V (1.6 ng/ml). They could not be detected by the IV–*II method either. Since we did not add detergent before this assay, the mEH in these supernatant may have been folded in a shape which is undetectable by the IV–*II assay. We speculated that this folding is somehow different from that of M-mEH in that the type IV epitope is hindered inside the mEH molecule in the supernatant and undetectable by the IV–*V method.

For further analysis, we concentrated the supernatants of THLE-5b, Huh-1 and LN-71 cultures about 10-fold by ultrafiltration through 10 kDa cut-off membranes. Then aliquots of these concentrates as

Table 5
Epitope selectivities of five types of monoclonal antibodies.

Type I	N-terminus (aa.21–143) linear epitope, Cross-reacts with a 54-kDa protein
Type II:	N-terminus (aa.21–143) linear epitope
Type III:	N-terminus (aa.54–71) linear epitope
Type IV:	C-terminus (aa.327–353) linear epitope
Type V:	Conformational epitope

Table 6
mEH detection by antibody sandwich methods.

Five kinds of monoclonal antibodies were labeled with ^{125}I or horse radish peroxidase (denoted by the asterisk), and used for detection of the purified mEH antigens (membrane-bound form of mEH: M-mEH or linearized form of mEH: L-mEH) which had been captured by the unlabeled homologous or heterologous antibodies (without asterisk) coated on the wells of 96-well plates. Signal/noise (S/N) ratios at 40 ng/ml of the antigen concentration are shown, and those above 10.0 are highlighted in gray.

RIA			ELISA		
Ab combinations	Antigen	S/N ratio	Ab combinations	Antigen	S/N ratio
II–*IV	M-mEH	1.8	II–*V	M-mEH	36.6
	L-mEH	1.8		L-mEH	N.D.
III–*IV	M-mEH	1.4	III–*V	M-mEH	20.2
	L-mEH	1.8		L-mEH	N.D.
IV–*II	M-mEH	2.5	IV–*V	M-mEH	48.4
	L-mEH	15.4		L-mEH	N.D.
IV–*III	M-mEH	2.0	V–*V	M-mEH	52.7
	L-mEH	9.0		L-mEH	N.D.
V–*V	M-mEH	15.4	IV–*II	M-mEH	1.9
	L-mEH	<1.0		L-mEH	15.7

N.D.: not determined.

well as the standard antigens, M-mEH and L-mEH, were subjected to ultrafiltration through 100 kDa cut-off membranes, and the flow-through fractions were obtained. When we measured these samples by the three antigen detection ELISA methods, the media of THLE-5b did not show positive signals. On the other hand, the mEHs in the supernatants of Huh-1 and LN-71 were well detected by V–*V, and we found that the detected antigens did not pass through the 100 kDa cut-off membranes indicating that they were in the shape of a multimolecular complex larger than 100 kDa (Fig. 6A). However, in contrast to M-mEH, the mEHs in the supernatants of Huh-1 and LN-71 could scarcely be detected by IV–*V (Fig. 6B) as was observed for the unconcentrated supernatants (Table 7). When we tested these samples by IV–*II without adding SDS, only L-mEH could be detected (data not shown). After addition of SDS, the antigens in the supernatants of the two cell lines, M-mEH as well as L-mEH could be detected, and we found that part of the antigens in the supernatants and most of L-mEH passed through the 100 kDa cut-off membranes (Fig. 6C). We could also see positive signals from the supernatant of THLE-5b especially after ultrafiltration through the100 kDa cut-off membrane. In addition, all of the three culture media showed significant mEH activity (0.025–0.046 nmol min^{-1} mg^{-1}). These results suggested that all of the three cell lines secrete a small amount of mEH with the size smaller than 100 kDa probably existing as single molecules folded in particular shapes which could not be detected by either of the IV–*II (without detergent treatment), V–*V, or IV–*V method. The majority of the mEH in the supernatants of Huh-1 and LN-71were in the shape of a multimolecular complex which was different from that of M-mEH and could not be detected by IV–*V.

Discussion

In this study, we demonstrate that mEH in some cancerous cells takes a different shape from that of the membrane-bound form of mEH and is released to the culture medium. To analyze the antigenic structure of mEH, we developed monoclonal antibodies that recognize different parts of the mEH molecule. When we tested sera from the immunized mice by ELISA using mEH fragments as the antigens, we found that the antibody against the C-terminal half (aa 244–455) was produced only after the third immunization, while the antibody against the N-terminus (aa 1–243) appeared after the first immunization, indicating that the C-terminal half is less immunogenic than the N-terminus. We developed the monoclonal antibody 6E3 against the epitope in the C-terminal half (aa 327–353) (type IV epitope) by hyperimmunizing mice with the purified mEH. In addition, we could obtain at least four kinds of monoclonal antibodies which recognize the N-terminus or the conformational epitopes (Tables 3, 5).

To obtain antibodies against the linear epitopes of mEH, we used L-mEH for immunization. L-mEH was prepared by preparative SDS-PAGE and does not react with the antibodies against conformational epitopes as shown by ELISA (Fig. 2). However, the antibodies from 26 out of 64 hybridoma colonies reacted only with the S-mEH but not with any of F-mEH 1 to 4. From these 26 hybridomas, 2G2 and 7B11 were obtained after limiting dilution, grouped as type V and shown to recognize conformational epitopes (Fig. 2). Therefore, it seems that L-mEH regained conformational epitopes after being injected into mice. The S-mEH, which was purified after solubilization with Triton X-100 and used for screening of hybridomas, did not react with either of the type V antibodies nor anti-AN antibodies just after purification, but during storage at 4 °C, it gained the reactivity with these antibodies (data not shown). These findings are further supported by the fact that the purified mEH exists as high molecular weight aggregates (~600 kDa) in the absence of SDS (DuBois et al., 1979; Lu et al., 1975). In addition, all of the 12 monoclonal antibodies raised against AN antigen (Akatsuka et al., 1986b) were found to recognize conformational epitopes of mEH (Akatsuka et al., 1986a), and in CBA against the AN antigen, all antibodies showed 100% inhibition to each other (unpublished). One of the anti-AN antibodies (1H9) and the type V antibody 2G2 compete with each other well in the assay against the M-mEH (Table 4). Taken together, it seems that mEH is prone to form a multimolecular complex with a common conformational epitope. Any of the monoclonal antibodies did not show significant inhibition of mEH catalytic activity. It is rare to find an inhibitory antibody probably because the active site of mEH is deep inside the enzyme which forms a peculiar conformation and complex.

In Western blotting of extracts from THLE-5b and Huh-1, the three antibodies (2D8, 5D8, and 8F11) reacted with a 54 kDa band in addition to the 47 kDa band, which corresponded to an entire mEH molecule (Fig. 3B), and were grouped as type I (Table 3). Since this 54 kDa band was not observed on the blots of Sf9 cells expressing mEH by infection with a recombinant baculovirus (Fig. 3A) or BHK-21 cells expressing mEH by transfection with an expression plasmid (Akatsuka et al., 2007) (data not shown), we think this is a protein distinct from mEH and was detected by a cross-reaction. A similar cross-reaction has been reported for anti-rat mEH monoclonal antibody, 25A-3 (Ananthanarayanan et al., 1988). 25A-3 recognized a 54 kDa protein which was thought to be the Na^+-independent bile acid carrier protein, but the antigen has not been molecularly identified (D. Levy, personal communication). Because mEH is thought to be a part of a multi-protein transport system on the membranes

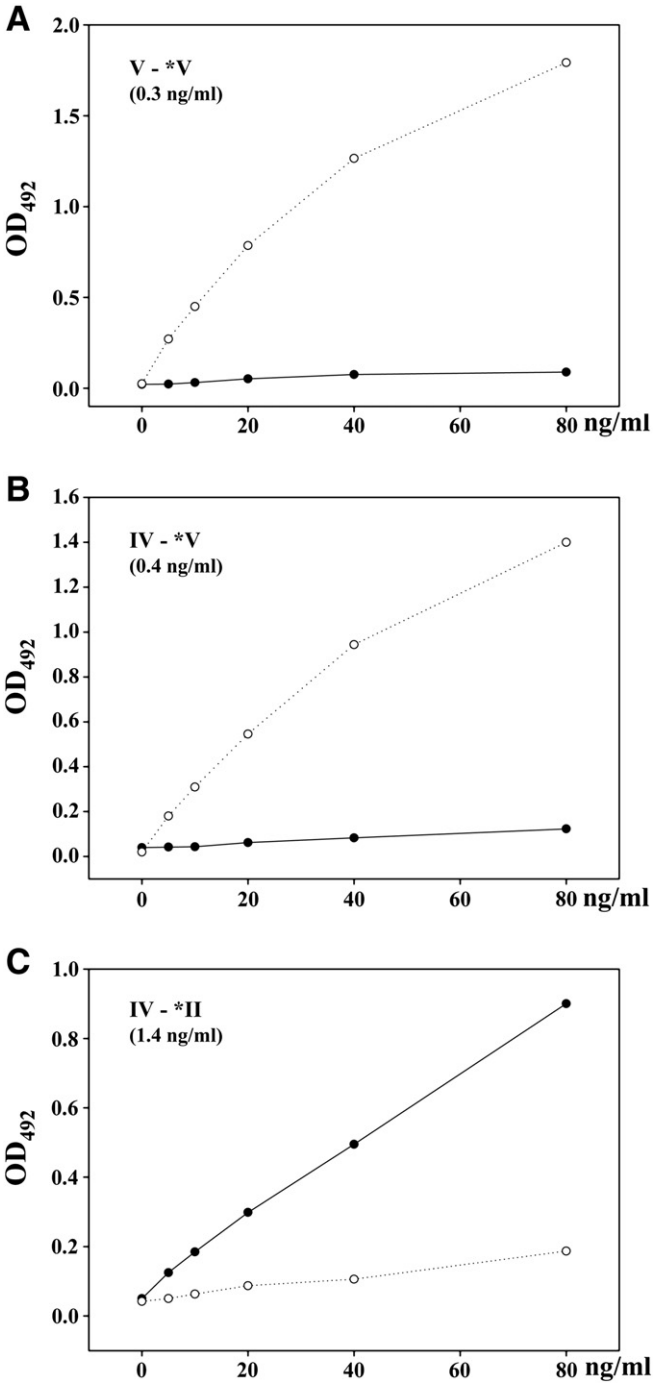

Fig. 5. Titration curves of the standard mEH antigens by antibody sandwich ELISA. Results of the three combinations of labeled (denoted by the asterisk) and unlabeled (without the asterisk) monoclonal antibodies (V–*V (A), IV–*V (B), and IV–*II (C)) are shown. ELISA plates coated with unlabeled antibodies at a concentration of 5 μg/ml were incubated with serial 2-fold dilutions of M-mEH (-○-) or L-mEH (●). After washing, the plates were incubated with optimal dilutions of HRP-labeled antibodies and washed. The color was developed by adding *o*-phenylenediamine dihydrochloride substrate and measured at an optical density of 492 nm. Cut-off values which correspond to the mean + 5 standard deviations of buffer control are shown in parentheses.

(Ananthanarayanan et al., 1988), it is worth identifying the 54 kDa protein in human cells recognized by type I antibodies to explore its possible relationship to mEH.

When we tested several combinations of the antibody in the development of sandwich assay methods for mEH antigen detection, M-mEH could be detected by the combination between the type V antibody and one of the type II to V antibodies (Table 6). This indicates that M-mEH expresses all of type II to V epitopes on its surface, however, it could not be detected by the combinations between the two of the type II to IV antibodies. One possible explanation is that the linear epitopes, type II to IV, are clustered in a very small area surrounded by the complex of mEH molecules with multiple type V epitopes, and once an antibody molecule binds to one of the linear epitopes, the other linear epitopes are hindered sterically from the access by another antibody molecule. On the other hand, L-mEH could be detected well by IV–*II, but the opposite combination II–*IV did not work well. In the epitope mapping study by ELISA using the antigens F-mEH 1 to 9, the type IV antibody 6E3 showed much lower reactivity to F-mEH 5 than to F-mEH 6 although F-mEH 5 contained the entire sequence of F-mEH 6 and an additional sequence on its C-terminal side (Table 2). This finding implies that the type IV epitope has the potential to be masked by the adjacent sequence located on its C-terminal side. Therefore, once the linearized mEH (L-mEH) is bound to the type II or type III antibody or to the plastic plate, it may change its conformation on the C-terminal side and type IV epitope may lose the accessibility to the antibody. This may also explain why the mEH released from Huh-1 and LN-71 to the culture medium could not be detected by the IV–*V method. The mEH in those cells may have changed its orientation on the membrane and formed a multimolecular complex with increased solubility and with the type IV epitope being masked by the adjacent sequence.

Table 7
mEH detection in culture supernatants by RIA. mEH in the culture supernatant was assayed by RIA with the three combinations of labeled (denoted by the asterisk) and unlabeled (without the asterisk) monoclonal antibodies and the purified mEH as the standard (L-mEH for IV–*II and M-mEH for IV–*V and V–*V). Results are expressed as ng/ml and values above the cut-off are highlighted in gray. Cut-off values were 2.1 (S/N ratio) using culture media as the negative control.

	Combinations of antibodies		
Cell line	IV–*II	IV–*V	V–*V
THLE-5b	<1.8	<1.6	<1.3
Huh-7	<1.8	<1.6	<1.36
Huh-1	<1.8	<1.6	4.26
M1	<1.8	<1.6	<1.3
U87MG	<1.8	<1.6	<1.3
LN-Z308	<1.8	<1.6	<1.3
LN-71	<1.8	<1.6	7.83

In this study, we found that mEH is released from Huh-1 and LN-71 to the culture medium and both antigens showed similar results in our analyses which demonstrated that they have a different structure from the membrane-bound form of mEH. This antigen seems to be related to the preneoplastic antigen (PNA). Okita and Farber (1975) demonstrated that there is an antigen in preneoplastic foci in rat livers that is released into the blood, which was in later studies shown to be similar if not identical to the mEH (Levin et al., 1978). In later studies, a rapid radiochemical assay for the PNA was developed for human blood and shown to be associated with liver cancer, but it was also found to be associated with several other types of liver damage (Hammock et al., 1984). The AN antigen was purified from liver of a patient infected with HCV (Tohmatsu et al., 1985). The antigen is released into the blood in the early phase of HCV infection and the antibody appears in the acute phase of hepatitis (Akatsuka et al., 1986a, 1986b, 2007). The AN antigen is composed of particles with molecular weight of more than 1500 kDa (Tohmatsu et al., 1985) and was found to be mainly composed of mEH (Akatsuka et al., 2007). However, the AN antigen was shown to be somehow different from the membrane-bound form of mEH in that the former reacted only with the antibody in sera from HCV-infected patients whereas the latter reacted with both the antibody from patients with HCV infection and that from patients with hepatitis A virus infection (Akatsuka et al., 2007). In this study, we did not analyze the AN antigen or the antigens in sera from patients with HCV

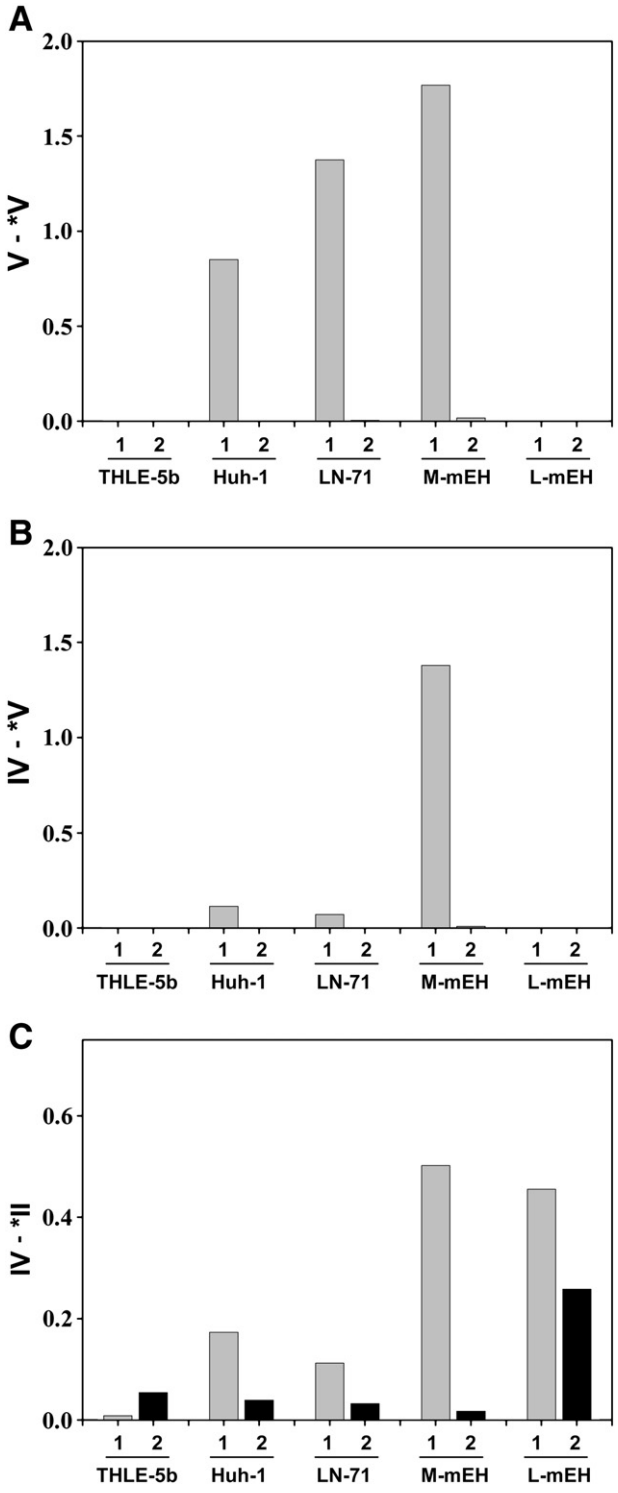

Fig. 6. Analysis of molecular sizes of mEH antigens released into the medium from THLE-5b, Huh-1 and LN-71 cultures. Culture supernatants were concentrated about 10-fold by ultrafiltration through 10 kDa cut-off membranes. Then, their aliquots and standard antigens (M-mEH and L-mEH, 80 ng/ml each) were subjected to ultrafiltration through 100 kDa cut-off membranes. The samples before (1) and after (2) ultrafiltration through 100 kDa cut-off membranes were tested by the three antigen detection ELISA methods (V–*V (A), IV–*V (B), and IV–*II (C)). In the assays by IV–*II (C), all the samples were suspended in the dilution buffer containing 0.1% SDS. The background-subtracted results are expressed by OD_{492}.

infection, but such studies will provide important information about the influence of virus infection on the expression and structure of mEH and its role in development of hepatitis.

Because mEH coordinates with other enzymes on ER membrane and plays multiple functions, disintegration of the enzymes may lead to pathological consequences such as tumorigenesis. We observed that mEH changes its location in the cells during certain virus infection in vitro, and it accompanies with the changes of mEH activities (manuscript in preparation). Further analysis of PNA-like antigens may clarify the association of mEH with the pathogenesis of some human diseases and cancer.

In summary, we have obtained monoclonal antibodies to at least five different epitopes of human mEH and developed the methods which selectively detect either the membrane-bound form or the linearized form of mEH. These methods enable us to discriminate the native form of mEH from the variant soluble form produced in some cancerous cells. The method which selectively detects the linearized form of mEH enables us to measure the mEH in any form of structure if only it has an entire sequence and linearized by SDS treatment. These tools may be valuable for elucidation of the role of mEH in various disease processes.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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