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Monoclonal antibodies reveal multiple forms of expression of human microsomal epoxide hydrolase

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ABSTRACT

In a previous study, we developed five kinds of monoclonal antibodies against different portions of human mEH: three, anti-N-terminal; one, anti-C-terminal; one, anti-conformational epitope. Using them, we stained the intact and the permeabilized human cells of various kinds and performed flow cytometric analysis. Primary hepatocytes and peripheral blood mononuclear cells (PBMC) showed remarkable differences. On the surface, hepatocytes exhibited 4 out of 5 epitopes whereas PBMC did not show any of the epitopes. mEH was detected inside both cell types, but the most prominent expression was observed for the conformational epitope in the hepatocytes and the two N-terminal epitopes in PBMC. These differences were also observed between hepatocyte-derived cell lines and mononuclear cell-derived cell lines. In addition, among each group, there were several differences which may be related to the cultivation, the degree of differentiation, or the original cell subsets. We also noted that two glioblastoma cell lines reveal marked expression of the conformational epitope on the surface which seemed to correlate with the brain tumor-associated antigen reported elsewhere. Several cell lines also underwent selective permeabilization before flow cytometric analysis, and we noticed that the topological orientation of mEH on the ER membrane in those cells was in accordance with the previous report. However, the orientation on the cell surface was inconsistent with the report and had a great variation between the cells. These findings show the multiple mode of expression of mEH which may be possibly related to the multiple roles that mEH plays in different cells.

Introduction

Microsomal epoxide hydrolase (EC 3.3.2.9) is a drug-metabolizing enzyme that catalyzes the conversion of epoxides formed during phase I metabolism of xenobiotics to trans-dihydrodiols (Newman et al., 2005). It is a highly hydrophobic nonglycosylated membrane protein and found in nearly all mammalian tissues. The highest mEH activity is observed in liver, with lower yet similar levels in testis, lung and heart (Waechter et al., 1988). In certain organs, the mEH is localized within specific cell types. For example, in cerebral tissues, mEH is primarily localized in glial cells (Teissier et al., 1998) and its activity is particularly high in tissues which function as blood- and cerebrospinal fluid-brain barriers such as the choroid plexus (Ghersi-Egea et al., 1994). In addition to the role in xenobiotic metabolism, mEH is implicated as a participant in endogenous steroid metabolism (Fandrich et al., 1995), and in the vitamin K reductase complex (Guenthner et al., 1998). mEH is known to be expressed on the plasma membrane and has been reported to act as a Na^+-dependent bile acid transporter (von Dippe et al., 1993). It is speculated that efficient execution of such multiple functions is secured by its orientation and association with P450 enzymes on the ER membrane and formation of a multiple transport system on the plasma membrane. Topological orientation of mEH has been determined by an N-glycosylation site tagging study, which revealed that the catalytic C-terminal domain faces the cytosol on the ER, and on the plasma membrane, the C-terminal faces the extracellular medium (Zhu et al., 1999).

In certain disease status, mEH loses its association with membrane and detected as a distinct antigen in the cytosol of neoplastic foci of liver (preneoplastic antigen; PNA) (Levin et al., 1978; Hammock et al., 1984; Okita and Farber, 1975), in the serum in association with

Abbreviations: mEH, microsomal epoxide hydrolase; ER, endoplasmic reticulum; M.F.I., mean fluorescent intensity; PNA, preneoplastic antigen; HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; PBS, phosphate-buffered saline; PBMC, peripheral blood mononuclear cells; PDI, protein disulfide isomerase; HCV, hepatitis C virus; BCIP, 5-bromo-4-chloro-3-indoyl-phosphate; NBT, nitroblue tetrazolium; HBs Ag, hepatitis B surface antigen.

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hepatitis C virus (HCV) infection (Akatsuka et al., 2007), or in some brain tumors (BF7/GE2 antigen) (Kessler et al., 2000). In the previous study, we have developed several anti-mEH monoclonal antibodies which should be grouped into the five types depending on their epitope selectivities (Duan et al., 2012). They comprised antibodies against N-terminal, C-terminal, and conformational epitopes. By combining these antibodies, we developed sensitive methods that could specifically detect either the membrane-bound form or the linearized form of mEH. These methods detected mEH in the culture medium released from a hepatocellular carcinoma (HCC) cell line Huh-1 and a glioblastoma cell line LN-71. These methods also revealed that the mEH in the culture medium had a different structure compared to the membrane-bound form of mEH. In this study, we applied these antibodies for the comparative analysis of the expression of mEH in various human cells including those derived from tumors. We also applied these antibodies for the determination of topological orientation of mEH on the membrane.

Materials and methods

Cell lines. THLE-5b, Huh-7, Huh-1, M1, U87MG, LN-Z308, and LN-71 have been described (Duan et al., 2012). LN-18, Raji, and Jurkat were obtained from the American Type Culture Collection (ATCC; Manassas, VA). LN-18 was cultured in DMEM with 10% FCS; Raji and Jurkat were cultured in RPMI1640 with 10% FCS.

Human liver and blood samples. Four samples were obtained from autopsies of four patients (#1–#4) conducted at the Department of Pathology, Saitama Medical University: #1 was a 79-year-old male who died of acute respiratory syndrome; #2 was a 74-year-old male who died of myocardial infarction; #3 was a 44-year-old female who died of cardiovascular collapse after an unsuccessful attempt at cardiac surgery which was accompanied by cholestasis and centrilobular hepatic necrosis; #4 was a 62-year-old male with fatty liver who died of acute pancreatitis. The fifth liver was obtained from a 74-year-old male patient (#5) at the partial hepatectomy for the treatment of metastatic colon cancer conducted at the Department of Digestive and General Surgery, Saitama International Medical Center. Plasma ALT levels were normal for patient #5 (19 IU/L), slightly increased for patients #1 (42 IU/L) and #2 (46 IU/L) and markedly increased for patients #3 (540 IU/L) and #4 (836 IU/L). The livers were put in cold PBS, sliced into 1-mm square pieces and digested in DMEM containing 10%FCS and 0.05% (w/v) collagenase (Wako Pure Chemicals, Osaka, Japan) at 37 °C for 30 min in the presence of 5% CO_2. The cells were passed through a 100 μm nylon mesh (BD Biosciences, Bedford, MA), washed (300×*g*, 5 min) twice and suspended in DMEM containing 10% FCS. Hepatocytes obtained were >95% viable as determined by trypan blue exclusion. PBMC were extracted from the venous blood of four healthy volunteers by centrifugation through a Ficoll–sodium metrizoate solution (LSM Lymphocyte Separation Medium; Cappel, Solon, Ohio).

Flow cytometric analysis. Adherent cells were grown to 80–90% confluency in 10-cm dishes (Falcon; BD Discovery Labware, Bedford, MA) and detached from the dishes by brief exposure to PBS containing 0.02% EDTA. Nonadherent cells were sampled from log phase cultures. The cells were washed once in PBS containing 2% FCS and 15 mM sodium azide (FACS buffer), then resuspended in FACS buffer at 2×10^7 cells/ml. For the cell surface staining, 50 μl of cells (1×10^6) was transferred to each well of a 96-well round-bottom plate, 50 μl of monoclonal antibodies (1:1000 dilution of ascites in PBS containing 10% FCS) were added, and the cells were incubated on ice for 30 min. The cells were washed three times with FACS buffer and incubated with FITC-labeled anti-mouse IgG F(ab′)2 fragment (Sigma-Aldrich, St. Louis, MO) for 30 min on ice. At the end of the incubation, cells were washed three times and resuspended in 100 μl of FACS buffer, and the stained cells were analyzed with a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The data were analyzed with CellQuest software (Becton Dickinson). Selective permeabilization of the plasma membrane was performed by suspending the cells in 4% paraformaldehyde-containing Cytofix/Cytoperm solution in the Cytofix/Cytoperm kit (BD Pharmingen, San Diego, CA) on ice for 30 min, washed twice with FACS buffer, then incubated in 25 μg/ml of digitonin (Sigma-Aldrich) in PBS on ice for 3 min. After washing twice with FACS buffer, the cells were stained in the same way as the cell surface staining. Permeabilization of the plasma membrane and ER membrane was carried out by treating the cells with Cytofix/Cytoperm solution and washing with FACS buffer (2×), then with a saponin-containing buffer (BD Perm/Wash buffer) in the Cytofix/Cytoperm kit (2×). The cells were suspended in Perm/Wash buffer at 2×10^7 cells/ml, and stained with the antibodies. Perm/Wash buffer was used for dilution of primary and secondary antibodies and washing of the cells. The integrity of ER membrane during the selective permeabilization experiments was confirmed by including the rabbit anti-protein disulfide isomerase (PDI) antibody (Sigma-Aldrich). The anti-HCV-core monoclonal antibody, 6G7 (IgG1) (Dubuisson et al., 1994) and the normal rabbit serum were used as the control antibodies for the monoclonal antibodies and the rabbit anti-PDI antibody, respectively. Mean fluorescent intensity (M.F.I.) for individual antibody staining was related to M.F.I. for the corresponding staining with the negative control antibody, and a relative M.F.I. value of 1.5 was used as cut-off for discriminating between positive and negative staining.

Western blotting. A membrane fraction of PBMC was separated with ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, La Jolla, CA), concentrated with 10% TCA, and suspended in SDS sample buffer. The protein content was measured with BCA assay (Pierce, Rockford, IL) using BSA as a standard, and 370 μg protein was applied into each lane of a 10% minigel (Mini-Protean TGX; Bio-Rad, Hercules, CA) and blotted to an Immobilon-P membrane (Millipore, Bedford, MA). The transferred antigen was detected with each type of monoclonal anti-mEH antibody IgG (10 μg/ml), anti-hepatitis C virus E1 (Dubuisson et al., 1994) (clone A4, IgG1) as the negative control, or anti-β-actin monoclonal antibody (Sigma-Aldrich) (1:5000 dilution) followed by peroxidase-labeled goat anti-mouse IgG (KPL, Gaithersburg, MA) and ECL Prime Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK). The image of the bands was scanned with Typhoon 9410 (GE Healthcare).

Results

mEH expression in human hepatocytes

Table 1 shows the monoclonal antibodies to human mEH developed in the previous study and grouped into five groups depending on their epitope selectivities (Duan et al., 2012). Using five antibodies, one from each type (type I: 5D8; type II: K4F8; type III: K2B7; type IV: 6E3; type V: 2G2), we examined the expression of mEH epitopes in various human cells by flow cytometry. At first, we obtained four liver samples (#1–#4) at autopsy and dispersed hepatocytes by collagenase digestion. When the cell surface was stained with the antibodies, all the epitopes except type IV could be clearly detected,

Table 1
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

among which type V epitope was most intensely stained (Fig. 1A). The results were similar among the three patients who did not have underlying liver diseases (#1 to #3), but the fourth patient with hepatic steatosis (#4) displayed type V epitope expression as much as 2-fold higher than the other three patients (Figs. 1A, C). To examine if the epitopes including type IV, which could not be detected on the cell surface, can be detected inside the cells, we obtained the fifth liver at a surgical operation of patient #5 and treated half of the hepatocytes with Cytofix/Cytoperm reagent which contains saponin and permeabilizes both the plasma membrane and the ER membrane (Wassler et al., 1987). When the permeabilized cells were stained with the antibodies, all of the five epitopes could be detected inside the cells, among which the type V epitope had the highest relative mean fluorescent intensity (M.F.I.) as it did in the cell surface staining (Figs. 1B, C). The type IV epitope was also detected inside the cells, but its intensity was relatively low. On intact cells, #5 showed a similar expression of mEH compared to #1–4 (Figs. 1A, C).

mEH expression in human PBMC

Next, we examined the expression of mEH in PBMC from four donors (#6 to #9). Although the enzyme activity (Seidegard et al., 1984), the gene expression (Krovat et al., 2000) of mEH in PBMC, and the protein expression (Krovat et al., 2000) of mEH in lymphoid cell lines have been demonstrated, the mode of mEH expression in these cells is not well known. In contrast to hepatocytes, we could not detect any of the epitopes of mEH on the surface of PBMC (Figs. 2A, C). On the other hand, inside the cells, type I, II, and IV epitopes were detected in significant amounts whereas type III and V epitopes were detectable in relatively small amounts (Figs. 2B, D). To confirm that PBMC express intact mEH molecules, we performed Western blotting of PBMC. We found that PBMC express a small amount of mEH of apparently the same size as that in the hepatocytes (Fig. 3).

Accordingly, we noticed striking differences of mEH expression between the two types of cells on the surface as well as inside of the cells. On the cell surface, hepatocytes express all of the epitopes except the type IV whereas PBMC express none of the epitopes. Inside the cells, the type V epitope is most abundant in hepatocytes whereas its expression is very low in PBMC.

mEH expression in hepatocyte-derived cell lines

Since we have found that the mode of mEH expression differs depending on the cell type, we wished to know whether the same type of cells alter the mEH expression depending on immortalization, cultivation, differentiation, or tumorigenicity. To this aim, we chose five hepatocyte-derived cell lines: THLE-3, THLE-5b, Huh-7, HepG2, and Huh-7. THLE-3 and THLE-5b were derived from normal human hepatocytes which were immortalized by transfection of SV40 T antigen gene. These cells maintain many functions of normal hepatocytes including phase II drug-metabolizing enzymes (Pfeifer et al., 1993). On the cell surface, the expressions of the five epitopes were almost absent (Figs. 4A, S1A). Type V epitopes were detected at very low intensity. Type II expression was also very low, but its positivity was not reproducible (Table 2, Fig. S3). On the other hand, significant signals were obtained inside the cells for all of the epitopes except type III (Figs. 4B, S1B). A noticeable observation was that the expression of type III and type IV epitopes inside the cells was inverse between

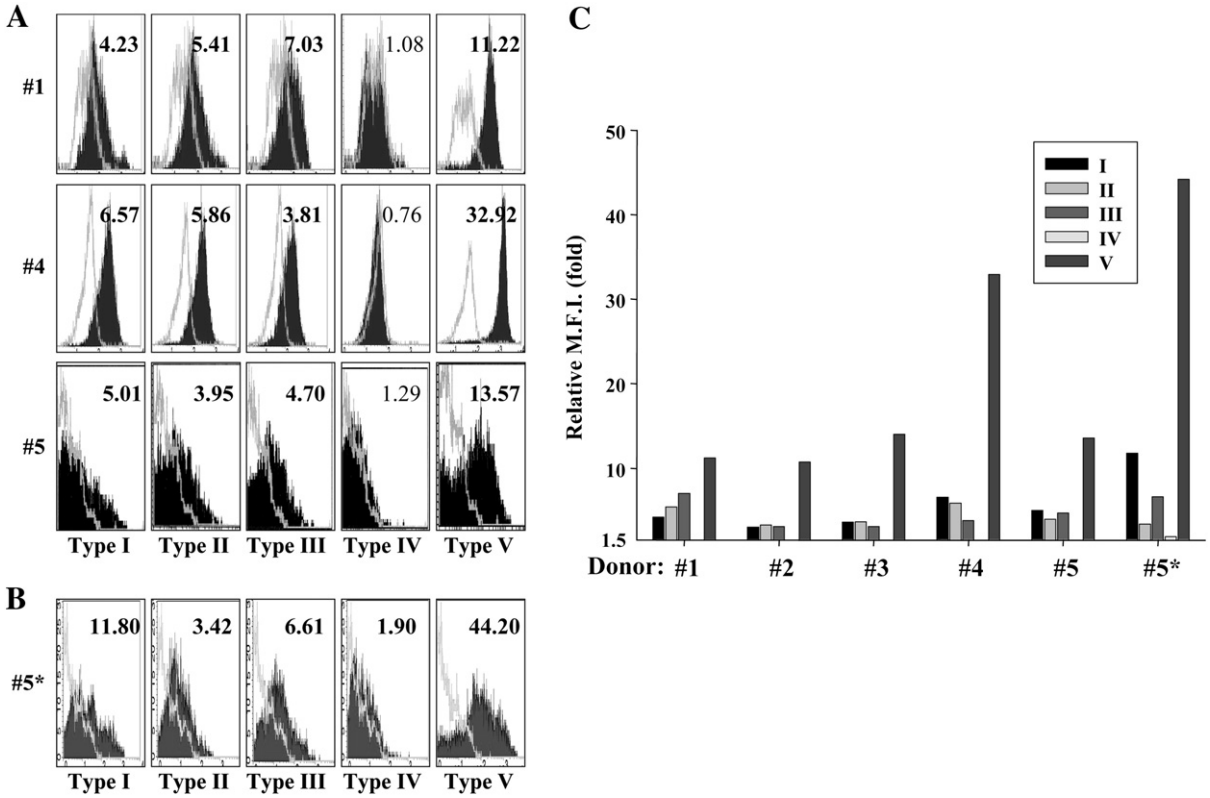

Fig. 1. Expression of the five types of mEH epitopes on the surface and inside of human primary hepatocytes determined by flow cytometry. A, Intact hepatocytes from the five patients (#1–#5) were stained with the five types of anti-mEH monoclonal antibodies and analyzed with a FACScan flow cytometer (the data of #1, #4, and #5 are shown). Black and white indicate the staining with the anti-mEH antibody and the isotype control, respectively. The values shown in the upper right indicate the relative mean fluorescent intensity (M.F.I.) using the M.F.I. obtained with the isotype control antibody as the reference, and those above 1.5 are shown in bold. B, Hepatocytes from the fifth patient (#5) were fixed with paraformaldehyde-containing Cytofix/Cytoperm solution and then suspended in saponin-containing Perm/Wash buffer to permeabilize the plasma membrane and ER membrane before staining with the antibodies which were diluted with Perm/Wash buffer. The results are shown as in A. C, The results of the five liver samples are shown by the relative M.F.I.. The asterisk indicates that the hepatocytes were permeabilized to analyze the mEH expression inside of the cells.

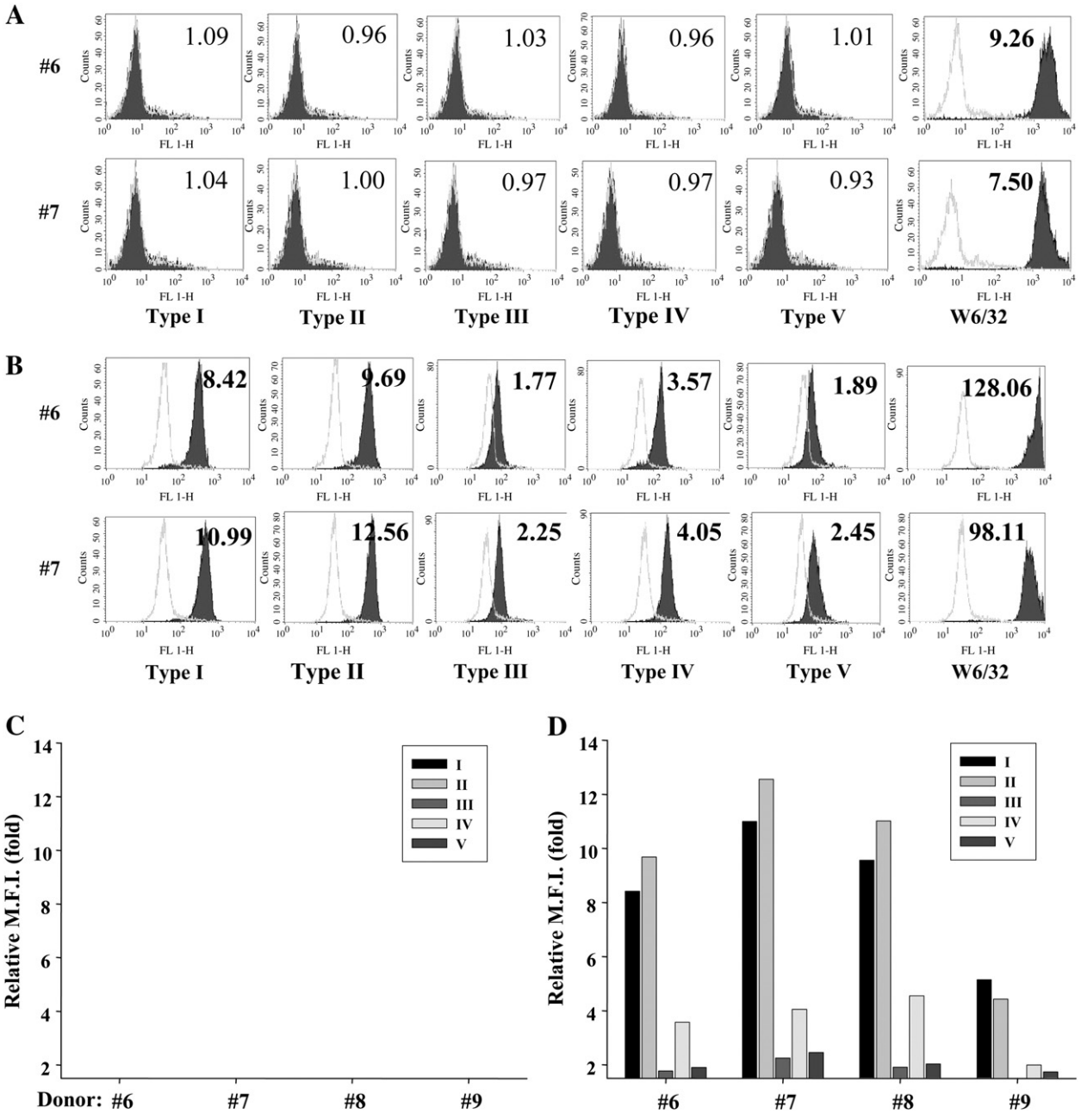

Fig. 2. PBMC from four healthy volunteers (#6 to #9) were analyzed for the expression of the five types of mEH epitopes on the surface and inside of the cells by flow cytometry. A, Intact PBMC were stained with the five types of anti-mEH monoclonal antibodies (black), anti-HLA class I monoclonal antibody W6/32 as the positive control (black), or isotype control antibody (white). The values shown in the upper right indicate the relative M.F.I. using the M.F.I. obtained with the isotype control antibody as the reference, and those above 1.5 are shown in bold. (the data of #6 and #7 are shown) B, Aliquots of PBMC were permeabilized and stained as performed for primary hepatocytes (Fig. 1) and the results of the donors #6 and #7 are shown as in A. C, The expression of the five mEH epitopes on the surface of PBMC from the four donors is shown by the relative M.F.I.. D, The expression of the five mEH epitopes inside of PBMC from the four donors is shown by the relative M.F.I.

the primary hepatocytes and the hepatocyte cell lines. In the primary hepatocytes, the type IV epitope was the least detectable while the type III epitope was the least in the hepatocyte cell lines. Huh-7 and HepG2 are well-differentiated HCC cell lines and secrete a variety of major plasma proteins; e.g., albumin, transferrin, alpha-fetoprotein, and the acute phase proteins. On the cell surface, these cells exhibited much higher expression of mEH epitopes (especially type V) compared to the normal hepatocyte-derived cell lines (Figs. 4A, S1A). mEH expressions inside the cells were almost the same as those of normal hepatocyte-derived cell lines with type V expression being the highest (Figs. 4B, S1B). Huh-1 is a HBs Ag-producing undifferentiated HCC line and produce tumors in nude mice. This cell line showed very high expression of all the epitopes on the cell surface (Figs. 4A, S1A) although their expression inside the cells was comparable to those by other four cell lines (Figs. 4B, S1B). Notably, the type IV epitope which was undetectable on the surface of primary hepatocytes or hepatocyte-derived cell lines was evidently detected on the surface of Huh-1.

mEH expression in mononuclear cell-derived cell lines

As we have noticed the differences of mEH expression profile between the primary hepatocytes and the hepatocyte-derived cell

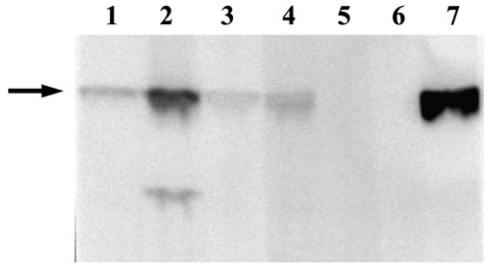

Fig. 3. Western blotting analysis of mEH expression in PBMC. The membrane fraction of PBMC was subjected to 10% SDS-PAGE, transferred to PVDF membranes, and immunoblotted with the type I (lane 1), II (lane 2), III (lane 3), IV (lane 4), V (lane 5) anti-mEH monoclonal antibodies, a negative control antibody (lane 6) or anti-β-actin antibody (lane 7).

lines, we next examined three lymphocyte-derived cell lines to compare with PBMC. Raji and Bjab were derived from patients with Burkitt's lymphoma and cell lines of B-cell origin which were found to harbor EB virus. Jurkat was derived from T-cell leukemia. On the cell surface, like PBMC, the three cell lines did not show any significant expression of any of the five epitopes except that the type II epitope was slightly detectable on Bjab cells (Figs. 5A, S2A). Inside the cells, all of the three cell lines showed significant expression of type II epitope (Figs. 5B, S2B). However, unlike PBMC, they did not show any signal for type III or IV epitopes. There was a big difference of type V epitope expression between the three cell lines. It was completely absent in Raji and Bjab, but it was high in Jurkat.

Accordingly, the mEH epitope expression profiles of the three mononuclear cell-derived cell lines were basically similar to but distinct from those of PBMC. They also showed differences between the two B-cell lines (Raji and Bjab) and a T-cell line (Jurkat).

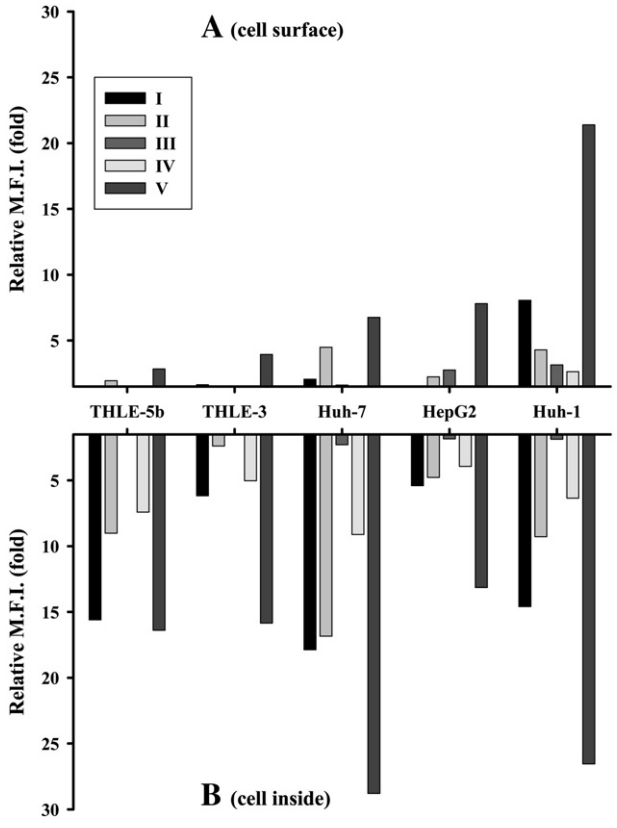

Fig. 4. Flow cytometric analysis of the expression of the five types of mEH epitopes on the surface (A) and inside (B) of the five hepatocyte-derived cell lines: THLE-5b, THLE-3, Huh-7, HepG2, and Huh-1. The cells were stained with anti-mEH monoclonal antibodies or isotype control antibody, and the relative M.F.I. are shown (the values above 1.5 are considered positive).

Table 2
Localization of the five types of mEH epitopes in the six cell lines determined by selective permeabilization and flow cytometric analysis. Intact cells or cells permeabilized with digitonin or saponin were stained with the five types of anti-mEH monoclonal antibodies, anti-HLA class I monoclonal antibody W6/32 as the positive control (not shown), anti-PDI antibody, or control antibodies (not shown), and the relative M.F.I. are shown (the values above 1.5 were considered positive and highlighted in gray).

		Anti-mEH monoclonal antibody					
Cells	Treatment	Type I	Type II	Type III	Type IV	Type V	Anti-PDI
M1	Intact	1.12	1.04	1.00	0.98	1.48	N.D.
	Digitonin	6.64	1.25	1.24	1.64	6.73	0.81
	Saponin	5.61	2.55	0.89	1.62	6.87	2.64
THLE-5b	Intact	1.11	0.97	0.95	0.97	1.92	N.D.
	Digitonin	15.78	1.20	1.41	4.00	17.66	0.70
	Saponin	10.64	3.06	0.84	2.74	21.58	1.75
U87MG	Intact	1.01	1.00	1.00	0.99	1.14	N.D.
	Digitonin	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	Saponin	3.32	1.53	0.81	1.60	3.55	5.10
LN-Z308	Intact	1.00	0.96	0.98	0.99	1.48	0.90
	Digitonin	7.54	1.04	1.24	2.58	26.87	1.36
	Saponin	7.19	2.07	0.87	3.24	24.30	1.89
LN-18	Intact	1.35	1.05	1.00	1.02	4.11	N.D.
	Digitonin	12.06	1.42	1.44	2.16	21.61	1.46
	Saponin	10.93	3.23	0.84	4.10	18.68	2.15
LN-71	Intact	1.23	1.02	1.02	1.08	6.65	0.95
	Digitonin	7.96	1.18	0.97	1.77	17.18	1.06
	Saponin	10.16	1.62	0.85	1.06	38.15	1.78

N.D.: not determined.

mEH expression in human glioblastoma cell lines

In the previous study, we have shown that the HCC line Huh-1 and the glioblastoma cell line LN-71 release mEH into the culture medium whereas other cell lines did not (Duan et al., 2012). It reminded us of 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). Actually, subcellular fractionation experiments of Huh-1 cells demonstrated that mEH in this cell line distributed widely to all the fractions including the soluble cytoplasmic fraction suggesting its loose association with the membrane (unpublished). Meanwhile, the mEH in some glioblastoma cell lines such as LN-71 have been described as antigenically different from other glioblastoma cell lines which was identified by the BF7/GE2 monoclonal antibodies (Kessler et al., 2000).

To assess the relationship between the BF7/GE2 antigen positivity and the expression of the five mEH epitopes, we performed flow cytometric analysis on the glioblastoma cell lines U87MG, LN-Z308, LN-18, and LN-71. LN-18 was also described as BF7/GE2-positive whereas LN-Z308 was as negative (Kessler et al., 2000). In these experiments, we included human fibroblast cell line M1 and a normal human hepatocyte-derived cell line THLE-5b for comparison. When the intact cells were stained with the antibodies, we noticed a prominent difference among the six cell lines (Table 2, Fig. S3: "Intact"). The two BF7/GE2-positive cell lines LN-18 and LN-71 exhibited marked type V epitope expression. On the other hand, THLE-5b showed weak signals for the type V epitope as was shown in the experiments described above (Figs. 4A, S1A) and other three cell lines including BF7/GE2-negative LN-Z308 did not show significant signals for any type of epitopes. When the cells were permeabilized and the inside of the cells was stained using the saponin-containing reagent, all of

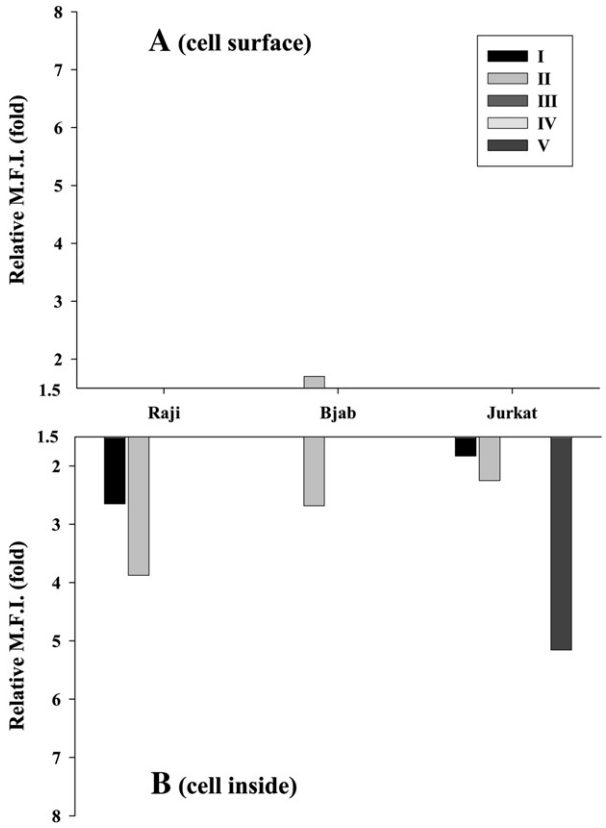

Fig. 5. Flow cytometric analysis of the expression of the five types of mEH epitopes on the surface (A) and inside (B) of the three mononuclear cell-derived cell lines: Raji, Bjab, and Jurkat. The cells were stained with anti-mEH monoclonal antibodies or an isotype control antibody, and the relative M.F.I. are shown (the values above 1.5 are considered positive).

the cell lines except LN-71 revealed essentially the same results: all of the mEH epitopes except type III were clearly positive (Table 2, Fig. S3: "Saponin"), while LN-71 did not show the type IV epitope signal at all inside the cells.

Taken together, the brain tumor-associated BF7/GE2 antigen positivity seemed to correlate with the surface expression of the type V epitope. This idea is also supported by the findings that Raji and Jurkat which had been described as BF7/GE2-negative (Schnegg et al., 1981) were also negative for the surface expression of the type V epitope (Figs. 5A, S2A).

Localization of mEH epitopes determined by selective permeabilization

In the experiments described above, we have shown that the mEH exists in various forms in various cells possibly depending on the cell type, cultivation, immortalization, differentiation, or tumorigenicity using the five antibodies which recognize different portions of mEH. Because they comprised antibodies against the N-terminal and the C-terminal portions of mEH, we used these antibodies to analyze the topological orientation of mEH in the cells. In the studies described above, permeabilization was performed using saponin-containing Cytofix/Cytoperm reagent in the Cytofix/Cytoperm kit which permeabilizes both the plasma membrane and the ER membrane. In the experiments shown in Table 2 and Fig. S3, we treated aliquots of the cells with digitonin which permeabilizes the plasma membrane leaving the ER membrane intact (Eriksson et al., 1991). To ensure that the permeabilization was carried out as intended, we included an antibody against the ER luminal protein, protein disulfide isomerase (PDI) as the control. As shown in Table 2 and Fig. S3 ("Digitonin"), anti-PDI antibody did not stain the cells demonstrating that the ER membrane was intact.

This selective permeabilization experiment demonstrated that all of the six cell lines had very similar topological orientation of mEH on the ER membrane: type I, IV, and V epitopes were detected on the cytoplasmic side of the ER membrane whereas type II epitope was detected on the luminal side of the ER membrane. The type III epitope was not detected in any of the six cell lines regardless of the pretreatments. As we confirmed the expression of the type III epitope in these cells by Western blotting (data not shown), the results shown in Table 2 and Fig. S3 suggested that this epitope is deeply embedded in the ER membrane or hidden in the molecular folding or multimolecular complex. Only LN-71 did not show type IV epitope signals after saponin treatment although they were detected in digitonin-treated cells. It is not clear why the permeabilization of the ER membrane leads to the loss of type IV epitope signals, but the results were reproducible in three independent experiments.

Discussion

In the study described in this report, we demonstrate that mEH displays various forms of expression. Because mEH plays multiple roles in different tissues we wished to obtain various primary human cells. Hepatocytes and PBMC were chosen because they were easier to obtain in large numbers compared to other cell types, and the mEH in the former cells was thought to act as bile acid transporter as well. Flow cytometric analysis revealed prominent differences between these two types of cells. Primary hepatocytes express 4 out of 5 types of mEH epitopes both on the cell surface and inside of the cells, among which, the type V was most abundant. On the other hand, PBMC did not show any of the five types of epitopes on the surface. They were found to express mEH inside the cells, however, the epitopes of the highest expression were types I and II, and type V epitope was only marginally positive. The mEH in PBMC was found to have the same molecular size as that in hepatocytes (Fig. 3). We also performed three kinds of antigen detection ELISA as described (Duan et al., 2012) for the lysate of PBMC and found that the mEH in PBMC did not take a multimolecular complex, but it could be detected by the antibody sandwich ELISA using type II and type IV antibodies after SDS treatment (data not shown). This means that the mEH has both the N-terminal and the C-terminal epitopes on the same molecule. Therefore, the mEH in PBMC seems to comprise the molecule of the same size and same antigenic structure as that of hepatocytes but takes different conformation and orientation on the membrane.

Compared to primary hepatocytes, normal hepatocyte-derived THLE-3 and THLE-5b exhibited very low surface expression of mEH epitopes. Inside the cells, their epitope expression profile was basically similar to that of the primary hepatocytes. The absence of surface expression of mEH was in accordance with the finding that rat hepatocytes lose their expression of mEH on the surface in 72 h of culture (von Dippe et al., 2003). HCC lines Huh-7 and HepG2 displayed significant levels of mEH expression on the surface, and the pattern of the expression of the five epitopes was similar to that of primary hepatocytes. Higher expression of mEH epitopes in HCC lines than in normal hepatocyte-derived cell lines is in accordance with the findings that mEH is sometimes up-regulated in malignant tumors (Coller et al., 2001; McKay et al., 1995), but the analysis specified on the cell surface expression has not been performed. An undifferentiated HCC line Huh-1 showed the highest expression of mEH epitopes on the cell surface. Our subcellular fractionation studies showed that in Huh-1 cells, mEH distributed throughout the fractions including the cytosolic soluble faction (data not shown). It suggested that the mEH in Huh-1 is somewhat similar to PNA, and in our previous study, we have shown that Huh-1 and a glioblastoma cell line LN-71 release mEH into their culture medium. The mEH released from

these two cell lines had distinct antigenic structure from that of the membrane-bound form of mEH: the mEH in the culture medium was not captured by the type IV antibody although the type IV epitope was detected after the mEH was linearized by SDS treatment (Duan et al., 2012). This finding suggested that the location of the type IV epitope on the mEH molecule is somehow altered in cancerous cells. This idea is further supported by the finding that the type IV epitope which was not detected on the surface of primary hepatocytes or other hepatocyte-derived cell lines was detected on Huh-1 (Figs. 4A, S1A). The type IV epitope expression in LN-71 was also different from that in other cell lines tested (Table 2, Figs. S3). In LN-71 cells, the type IV epitope was not detected in the luminal side of the ER although it was detected on the cytoplasmic side.

Mononuclear cell-derived cell lines Raji, Bjab, and Jurkat seemed to share the characteristics of mEH expression with PBMC. However, only Jurkat exhibited remarkable expression of type V epitope inside the cells (Figs. 5B, S2B). It is not clear whether this marked difference of type V epitope expression between the three cell lines reflects the difference of their origin or the factors involved in the establishment of these cell lines. Because PBMC showed very low signals for type V epitope, the small population of PMBC may have the same expression profile as Jurkat. Therefore, it is of interest to do this kind of analysis after separation of PBMC based on the cell surface markers.

Among the four glioblastoma cell lines tested, only LN-18 and LN-71, which had been reported as the BF7/GE2 antigen-positive, revealed significant signals for the type V epitope (Table 2, Fig. S3). As the BF7/GE2 antigen was identified in the screening of hybridomas by the antibody binding radioimmunoassay using living LN-18 cells as the antigen (Schnegg et al., 1981), we speculate that this antigen is expressed on the cell surface and related to the type V epitope.

Elevation of the type V epitope expression was also noted in the hepatocytes. The primary hepatocytes from patient #4 with liver steatosis showed the type V epitope signals higher than those from the other three patients who did not have underlying liver diseases (Fig. 1). The HCC line Huh-7 and Huh-1 also exhibited higher type V epitope expression than the normal hepatocyte-derived THLE-3 and THLE-5b (Fig. 4). In addition, we observed that the type V epitope expression was augmented in THLE-5b and Huh-7 when they were infected in vitro with hepatitis A virus or other viruses (unpublished data). These lines of evidence suggest that surface expression of mEH is altered in association with some pathogenic status, and may be involved in the pathogenesis of hepatitis or liver cancer.

Topological orientation of mEH has been determined by introducing an N-glycosylation site into an mEH-expressing plasmid and introducing it in COS-7 cells (Zhu et al., 1999). According to their study, the N-terminus and the C-terminus of mEH faces the luminal side and the cytoplasmic side of the ER membrane, respectively (type I topology), while the orientation on the plasma membrane is opposite (type II topology). However, this kind of approach is not applicable to a comparable study of mEH topology for various cells. We developed antibodies against N-terminal and C-terminal portions of mEH which enable us to assess the topological orientation of mEH on the membrane. Using them, we performed flow cytometric analysis on the six cell lines after selective permeabilization and could find consensus features (Table 2, Fig. S3). The type I epitope was found on the cytoplasmic side of the ER membrane whereas the type II epitope was on the luminal side. In a previous study, we have shown that the type I and the type II epitope overlap each other on the mEH, but the type I antibody recognizes the 54 kDa protein as well probably by cross-reaction (Duan et al., 2012). Taking this into account, we speculate that the N-terminal portion of mEH locates on the luminal side of the ER membrane while the 54 kDa protein locates on the cytoplasmic side. On the other hand, the type IV epitope which resides in the C-terminal half of mEH could be detected on the cytoplasmic side of the ER membrane. These findings were common among the six cell lines tested except LN-71, and in agreement with the type I topology previously reported.

In contrast to the orientation on the ER membrane, our findings on the orientation of mEH on the plasma membrane were not in accordance with a previous report (type II topology). On the surface of primary hepatocytes, the type II and the type III epitopes on the N-terminal side of mEH were detected whereas the type IV epitope on the C-terminal side of mEH was not (Fig. 1). Well-differentiated HCC lines Huh-7 and HepG2 showed a similar expression profile (Fig. 4). Furthermore, mEH topology on the cell surface seems different between the cells. The type III epitope was detected neither on the cell surface nor inside of the cell lines derived from multiple cell types (Fig. 5, Table 2), therefore, it seemed to be deeply embedded in the membrane or buried inside of the folding of the mEH molecule. On the other hand, this epitope was detected with significant signals on the surface of hepatocytes and HCC lines (Figs. 1, 4). The glioblastoma cell lines LN-18 and LN-71 showed the surface expression of mEH, but its expression profile was unique in that only the type V epitope was detected. Taken together, the mEH expression and its topological orientation on the surface of the cells seem to vary greatly and possibly regulated by unknown multiple factors.

In summary, we report that the mode of mEH expression varies greatly between the cells. Our findings indicate the necessity of intensive analysis on the relationship of the expression profile and the functions of mEH in the cells and the mechanisms which regulate this variety of expression.

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