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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