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Human glutamate dehydrogenase is immunologically distinct from other mammalian orthologues

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Abbreviations: hGDH, human glutamate dehydrogenase; mAbs, monoclonal antibodies; TBS, Tris-buffered saline

Abstract

Five monoclonal antibodies (mAbs) that recognize human glutamate dehydrogenase (GDH) have been selected and designated as monoclonal antibodies hGDH60-6, hGDH60-8, hGDH63-10, hGDH63-11, and hGDH91-14. A total of five mAbs recognizing different epitopes of the enzyme were obtained, two of which inhibited human GDH activity. When total proteins of human homogenate separated by SDS-PAGE, were probed with mAbs, a single reactive protein band of 55 kDa, which co-migrated with purified recombinant human GDH was detected. When the purified GDH was incubated with each of the mAbs, its enzyme activity was inhibited by up to 58%. Epitope mapping analysis identified, two subgroups of mAbs recognizing different peptide fragments. Using the individual anti-GDH antibodies as probes, the cross reactivities of brain GDH obtained from human and other animal brain tissues were investigated. For the human and animal tissues tested, immunoreactive bands on Western blots appeared to have the same molecular mass of 55 kDa when hGHD60-6, hGHD60-8, or hGHD91-14 mAbs were used as probes. However, the anti-human GDH mAbs immunoreactive to bands on Western blots reacted differently on the immunoblots of the other animal brains tested, *i.e.*, the two monoclonal antibodies hGDH63-10 and hGDH63-11 only produced positive results for human. These results suggest that human brain GDH is immunologically distinct from those of other mammalian brains. Thorough characterization of these anti-human GDH mAbs could provide potentially valuable tool as immunodiagnostic reagents for the detection, identification and characterization of the various neurological diseases related to the GDH enzyme.

Keywords: cross-reactivity; epitope mapping; human glutamate dehydrogenase; immunoblot; monoclonal antibodies

Introduction

Glutamate dehydrogenase (GDH) (EC 1.4.1.3) catalyzes the reversible deamination of 1-glutamate to 2-oxoglutarate using NAD^+ or $NADP^+$ as coenzyme (Smith *et al*., 1975). GDH has long been used as a marker for mitochondria in brain and other tissues. However, the activity of GDH is also enriched in the nuclear fraction as well as in the mitochondria fraction (Lai *et al*., 1986). Recent biochemical works led to the finding that multiple forms of GDH are present in mammalian system and that the activities of the GDH isotypes differ in their relative resistance to thermal inactivation, detergent extractability, and allosteric regulation characteristics (Plaitakis *et al*., 1984; 2000; Abe *et al*., 1992; Cho *et al*., 1995; Shashidharan *et al*., 1997). These forms have been designated soluble and particulate GDH (Plaitakis *et al*., 1984; Colon *et al*., 1986; Hussain *et al*., 1989). Although many studies show the subcellular localization of GDH (Schmitt and Kugler, 1999), the origin of the GDH polymor-

phism is not known. It was reported that the presence of four differently sized mRNAs and multiple gene copies for GDH occur in the human brain (Plaitakis *et al*., 1993). A novel cDNA encoded by an X chromosome-linked intronless gene also has been isolated from human retina (Shashidharan *et al*., 1994; 1997). The importance of the GDH-deficient neurological disorders has attracted considerable interest. Four different forms of GDH isoproteins were detected from the human cerebellum of normal subjects and patients with neurodegenerative disorders (Duvoisin *et al*., 1983; Plaitakis *et al*., 1984; Hussain *et al*., 1989). The GDH isoproteins are differentially distributed in the two catalytically active isoforms of the enzyme (Colon *et al*., 1986; Plaitakis *et al*., 1993). The enzyme isolated from one of the patients with a variant form of multisystem atrophy displayed a marked reduction of one of the GDH isoproteins (Hussain *et al*., 1989). However, it is not known whether the distinct properties of the GDH isoproteins are essential for the regulation of glutamate metabolism. At present, the functional significance and reaction mechanism of GDH isotypes in nerve tissue remains to be studied. Recently, we isolated two soluble forms of GDH isoproteins (designated GDHI and GDHII) from bovine brain, and the GTP binding site within the GDH isoproteins were identified by using photoaffinity labeling (Cho *et al*., 1995; 1996; 1998). The results from our recent studies in brain demonstrate that the bovine brain GDH isoproteins are different gene products rather than the results of posttranslational modifications (Cho *et al*., 1995; 1998). We also identified an essential lysine residue by using o-phthalaldehyde, pyridoxal 5'-phosphate (PLP) and peptide analysis (Kim *et al*., 1997; Ahn *et al*., 1999). More recently, we synthesized and expressed the human GDH gene and identified the GTP binding site by the cassette mutagenesis and photoaffinity labeling (Lee *et al*., 2001; Yoon *et al*., 2002).

In the present study, the recombinant human enzyme was injected as an immunogen into BALB/c mice to obtain monoclonal antibodies (mAbs), and five mAbs to the protein were produced from the fusion experiments. The mAbs, which specifically recognized human GDH on Western blots, were characterized and used as probes for a cross-reactivity study of the brain enzymes from human, and other mammalian sources. In addition, we identified the cross reactivities with various rat tissues and tumor cell lines.

Materials and Methods

Materials

NADH, bovine serum albumin, L-glutamate, EDTA, phenylmethylsulfonyl fluoride, 2-oxoglutarate, ADP, Sephadex G-100 and G-200 were purchased from Pharmacia LKB. ammonium acetate, 2-mercaptoethanol, and goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) were purchased from Sigma. Basal medium Eagle, Dulbecco's Modified Eagle's (DME) medium, hypoxanthine-aminopterin-thymidine (HAT), polyethylene glycol (PEG) for fusion and penicillin/streptomycin antibiotics were purchased from GIBCO BRL. Goat anti-mouse IgG conjugated with alkaline phosphatase (AP) and conjugated with horseradish peroxidase (HRP) was obtained from Santa Cruz. Dialysis tubing was purchased from Sigma. Hybond-C^+ nitrocellulose membrane and ECL western blotting detection reagent were purchased from Amersham. All other reagents were of the highest purity available.

Enzyme purification

Human GDH gene (pHGDH) has been chemically synthesized and overexpressed as a soluble protein in *E. coli* as previously described (Cho *et al*., 2001). *E. coli* BL21 (DE3) was used for high level expression of the recombinant human GDH. Purification of human GDH was performed with a slight modification of the previous methods (Cho *et al*., 1995).

Fresh overnight cultures of DE3/pHGDH at 37°C were used to inoculate 1 L of LB containing ampicillin. After the cells had grown until the A_{600} reached 1.0 and then IPTG was added to a final concentration of 1 mM, and the incubation was continued for 3 h. The cells were harvested by centrifugation. Cell pellets were suspended in 100 ml of 100 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM dithiothreitol and lysed with a sonicator. Cellular debris was removed by centrifugation and the crude extract was precipitated by 30~65% ammonium sulfate. After centrifugation at 12,000 *g* for 30 min at 4°C, the pellet was dissolved in a minimum amount of buffer A (2 mM potassium phosphate (pH 7.0), 1 mM EDTA, 5 mM dithiothreitol) and dialyzed against buffer A. The dialyzed sample was loaded onto a hydroxylapatite column (2.5×10 cm) (Bio-Gel HTP, Bio-Rad) that was equilibrated with buffer A. The column was washed with 10 mM potassium phosphate (pH 7.0), 1 mM EDTA, 5 mM dithiothreitol until the breakthrough peak of protein had been eluted. The enzyme was then eluted by a gradient up to 200 mM potassium phosphate. The fractions containing GDH were pooled, concentrated, and buffer changed to buffer B (20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5 mM dithiothreitol) using Amicon concentrator and then applied to a FPLC Resource-Q anion exchange column equilibrated with buffer B. The enzyme was then eluted using a linear gradient made with buffer B in increasing concentration of NaCl (from 0 mM to 100 mM) at 0.5 ml/min. The frac-

tions containing GDH were combined and applied to a HPLC Protein-Pak 300 SW gel filtration column (0.15×30 cm) equilibrated with buffer B and the proteins were eluted with the same buffer at 1 ml/min.

The purified GDH was analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and recognized by western blot using monoclonal antibodies against the bovine brain GDH previously produced in our laboratory (Choi *et al*., 1999). Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard. Since *E. coli* only has an $NADP^+$-dependent GDH (McPherson and Wootton, 1983), the enzyme assay was performed with NAD^+ as a coenzyme as described elsewhere (Cho *et al*., 1995).

Enzyme assay

GDH activity was measured spectrophotometrically at saturating conditions in the direction of reductive amination of 2-oxoglutarate by following the decrease in absorbance at 340 nm as described before (Cho *et al*., 1995). Briefly mentioned, all assays were performed in duplicate, and initial velocity data were correlated with a standard assay mixture containing 50 mM triethanolamine (pH 8.0), 100 mM ammonium acetate, 0.1 mM NADH, 2.6 mM EDTA, and 1 mM ADP at 25°C. GDH concentrations were adjusted to give a measured rate of less than 0.04 absorbance units per minute. The reaction started with the addition of 2-oxoglutarate to 10 mM final concentration. One unit of enzyme was defined as the amount of enzyme required to oxidize 1 mol NADH/min at 25°C.

Production of anti-human GDH mAbs

For injection, the purified recombinant hGDH (100 g in a volume of 200 l) was mixed with an equal volume of Complete Freund's Adjuvant by sonication for three 15-s bursts at 30% maximum intensity. The antigen-adjuvant mixture was injected into a female BALB/c mouse (6-8 weeks old) intraperitoneally. The first injection was followed by three booster injections at 2 to 3-week intervals. The final injection was given 3 days before the cell fusion without adjuvant. The feeder layer cells were prepared 1 day before fusion. A 12-18 weeks old BALB/c mouse was killed by cervical dislocation, and its abdominal skin was removed carefully. Five milliliters of ice-cold 11.6% sucrose solution was injected into the peritoneal cavity, ~3 ml of the injected solution was pulled out, and peritoneal cells were collected by centrifugation for 5 min at 650 *g*.

The fusion experiments were performed as follows (Choi *et al*., 1995; 1996). In brief, spleen cells released by tearing the removed spleen with fine forceps were collected in a 15-ml centrifuge tube. Then the prepared spleen cells and SP2/o-Ag-14 mouse myeloma cells (Shulman *et al*., 1978) were combined, and 1 ml of 50% polyethyleneglycol 1500 in DME (serum free) was added slowly. The fusion process was allowed to continue for 90 s at 37°C and stopped by adding DME. To avoid an osmotic shock, 1 ml of DME was added slowly for the first 1 min, and 2 ml was added for the next 1 min. For a period of 10 min, a total of 20 ml of DME was added. The cells were collected by centrifugation for 1 min at 650 *g*, suspended in 20 ml of selective HAT medium (DME supplemented with 20% fetal bovine serum, antibiotics, and HAT) carefully by swirling, and centrifuged for 1 min at 650 *g*. The cells were resuspended in 120 ml of HAT medium, and 200 l of cell suspension was transferred into each well of six 96-well plates. About 2 weeks after the fusion, culture supernatants were collected and first screened by immunodot-blot analysis with purified enzyme as an antigen and then by western blot analysis. Positive clones selected by the screening methods were transferred to six-well plates, grown in tissue culture flasks (75 cm^2), and frozen in liquid nitrogen tank. All positive clones were frozen first and cloned by limiting dilution after thawing.

Cloning by limiting dilution to isolate a single specific antibody-secreting cell

For cloning of a single specific antibody-secreting cell, aliquots of cultured cells were diluted in fresh DME medium and counted using haematocytometer. Samples to be cloned were diluted in hypoxanthine-thymidine (HT) medium to 10 cells/ml. 100 l of well-suspended sample was plated in each well of 96-well plate and 100 l of fresh HT media were added. It was fed at day 5 and day 12 with two drops of medium. The cells of each well were expanded and reassayed by western blot analysis.

Immunoblot analysis

For immunodot-blotting, small squares (1×1 cm) were drawn on a sheet of nitrocellulose paper (10× 10 cm) and marked by numbering. One microliter of antigen solution (0.5 mg/ml) was applied onto each square and air-dried. The blots were incubated for 1 h in Blotto [2% nonfat dry milk in Tris-buffered saline (TBS)], rinsed briefly with TBS, and air-dried. The blots were processed by the procedures described in Western blotting. For Western blotting, proteins separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were electrophoretically transferred to nitrocellulose membranes (Towbin *et al*., 1979), and the membranes were rinsed briefly in distilled water and air-dried. The blots were blocked with Blotto for 1 h at 37°C. After rinsing with TBS, the blots were

incubated in culture supernatants for 1 h and washed three times in TBS containing Tween 20 at 5 min intervals. Then, the membrane was incubated for 1 h at 37°C with horseradish peroxidase conjugated goat anti-mouse IgG antibodies, diluted 1:5,000 in TBS containing 0.05% Tween-20. Finally, the bound conjugate was identified by incubation of the membrane in substrate buffer (0.5 mg/ml 4-chloro-1-naphthol in 1:5 v/v methanol/TBS and 0.015% H_2O_2) for 5 min at room temperature.

Purification of mAbs

For purification of mAbs, 100 ml of the culture supernatant was centrifuged for 30 min at 15,000 *g* to clarify cells and insoluble aggregates and applied onto a protein A-agarose column (packed volume, 1 ml; Amersham Phamacia). The column was washed with phosphate-buffered saline (PBS) until the absorbance of unbound proteins came down to the background level, and antibodies were eluted with 0.1 M glycine-HCl (pH 2.5). The eluted antibodies were neutralized by addition of 1 M Tris and dialyzed against 10 mM Tris-HCl (pH 6.5).

Enzyme-inhibition test

The effects of the mAbs on the inhibition of GDH activities were investigated by measuring catalytic activity of the GDH (5 g) after pre-incubated with purified monoclonal antibody (50 g) for 1 h at 37°C. Enzymatic assays of the samples were measured by the method described in the "Enzyme assay".

Epitope mapping

One-dimensional epitope mapping was carried out according to a procedure previously described (Choi *et al*., 1995; 1996). Ten micrograms of purified human GDH in SDS sample buffer were mixed with an equal volume of *Staphylococcus Aureus* V-8 protease solution (0.3 g in SDS sample buffer). The mixtures were assayed by SDS polyacrylamide gel and the separated peptides were transferred for immunoblotting analysis as described above.

Cell culture

Mouse neuronal cell line PC12, mouse macrophage J774A1, human hepatocellular carcinoma HepG2 (ATCC No. HB-8065), human squamous cell carcinoma SiHa (ATCC No. HTB17), and human cervical adenocarcinoma HL3T1 were obtained from Korean Cell Line Bank. All cell lines were grown in DME containing 10% FBS according to the supplier's instruction.

Cross reactivities among animal brains including human and tumor cell lines

Several animal brains from cow, pig, dog, cat, rat and chicken were removed and homogenized in 10 mM potassium phosphate buffer containing 0.1 mM EDTA, 1 mM 2-mercaptoethanol and 1 mM PMSF. The individual 25% (w/v) homogenates were centrifuged at 10,000 *g* for 1 h. Five microliters of each supernatant were mixed with an equal volume of 2×SDS sample buffer and boiled for 3 min. The cooled samples were applied to SDS-PAGE and transferred to nitrocellulose membranes. After several cell lysis from various tumor cell lines such as PC12, J774A1, HepG2, SiHa, and HL3T1 were transferred nitrocellulose membranes. The blots were processed by the procedures described in Western blot. In the case of human brain, total proteins were prepared by homogenizing a small fragment of cerebral cortex removed from a 45-year old male who required a surgery after an accident.

Cross reactivities among rat organs

Several rat organs including brain, heart, lung, liver, stomach, testes, and skeletal muscle removed and homogenized in 10 mM potassium phosphate buffer containing 0.1 mM EDTA, 1 mM 2-mercaptoethanol and 1 mM PMSF. The individual 20% (w/v) homogenates were centrifuged at 10,000 *g* for 1 h. The samples were applied onto a 10% SDS polyacrylamide gel and the separated peptides were transferred for immunoblotting analysis as described above.

Other methods

Discontinuous SDS-PAGE was carried out as described by Laemmli (1970). Spectrophotometric measurements were carried out with a Kontron model UVIKON 930 double-beam spectrophotometer.

Results

Purification of the gene product

GDH encoded by pHGDH in DE3 was purified by several chromatographic methods (see Materials and Methods). As the recombinant human GDH was readily solubilized, no detergents were required throughout the entire purification procedure. The purified GDH was estimated to be $>$ 98% pure by SDS/PAGE (Figure 1A). The subunit size (55 kDa) and the native size (320 kDa) of the hexameric recombinant GDH was determined by SDS/PAGE and HPLC gel filtration chromatography (data not shown), respectively. Purified GDH protein was also similar to those of other authentic GDHs (Smith *et al*., 1975; Hussain *et al*., 1989; Cho *et al*., 1995).

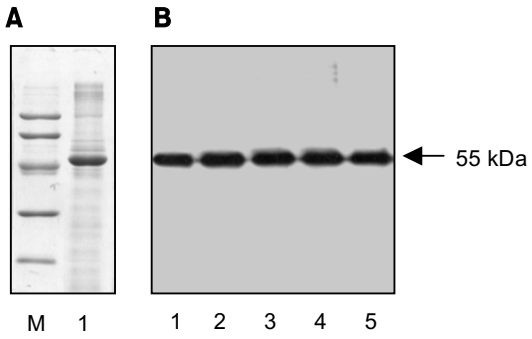

Figure 1. Expression and purification of protein (A) and corresponding immunoblots (B) of purified human GDH probed with representative hGDH mAbs: lane 1, hGDH63-10; lane 2, hGDH63-11; lane 3, hGDH60-6; lane 4, hGDH60-8; lane 5, hGDH91-14. A total of 104 were initially selected by immuno dot blot analysis. All of them recognized the purified human GDH.

Production of mAbs

Human GDH protein, which was expressed and purified using several stages of column chromatographies, exhibited a single protein band on SDS-polyacrylamide gel (Cho *et al.*, 2001). To enhance the immunogenicity of the protein and to obtain antibodies with a better reactivity on Western blot, purified enzyme was denatured in the presence of SDS and injected into animals. From two fusion experiments, 104 positive clones were initially screened by immunodot-blot analysis. Because goat anti-mouse IgG antibody was used as a second antibody, all monoclonal antibodies screened were of the IgG classes. Among the 104 clones hybridomas, some clones had lost the ability to produce monoclonal antibodies that reacted with the IgG protein on western blot, and thus were discarded. Twenty-four hybridomas of the 96 clones were finally selected for further study. The immunoreactivities of some representative mAbs for purified human GDH are shown in Figure 1B.

Characterization of mAbs

To check the specificities of the mAbs, total human brain protein was extracted, separated by SDS-PAGE, and immunoblotted with produced the mAbs. The antibodies specifically recognized a protein band of 55 kDa on SDS-PAGE (Figure 2A), which corresponded to the position of purified GDH. To determine if the mAbs inhibit GDH activity, samples of the purified enzyme were incubated with the different mAbs. Of the five representative monoclonal antibodies tested, two mAbs (hGDH63-10, hGDH63-11) inhibited enzyme activity, and the maximum extent of this inhibition observed was 58%. However, no inhibition was observed following treatment with the other

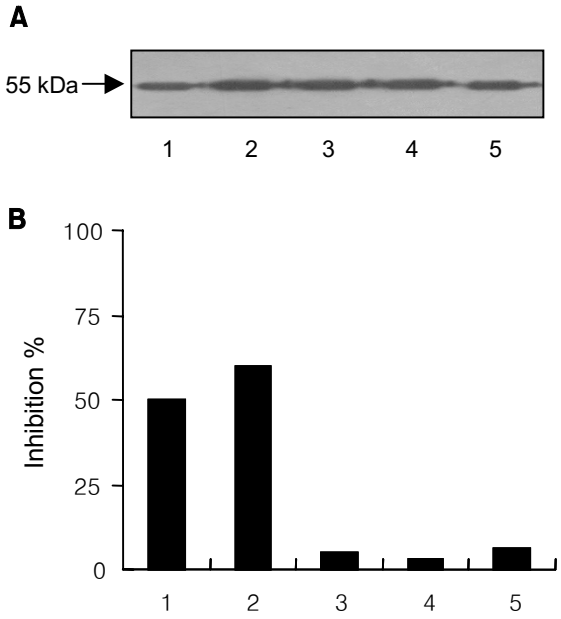

Figure 2. An immunoblots of total proteins of human brain homogenate (A) and inhibition of GDH activity by the purified mAb (B): lane 1, hGDH63-10; lane 2, hGDH63-11; lane 3, hGDH60-6; lane 4, hGDH60-8; lane 5, hGDH91-14. All mAbs specifically recognized a protein band of 55 kDa. Purified human GDH (1 g in 10 l of 0.1 M potassium phosphate, pH 7.2) was incubated with purified mAbs (10 g in 90 l PBS) for 1 h at room temperature. Then, the enzyme activities of the samples were determined as described in materials and methods. As a control, fresh culture supernatant was used, one of five mAbs (hGDH63-11) inhibited GDH activity up to a maximum of 58%.

three mAbs (hGDH60-6, hGDH60-8 or hGDH91-14) (Figure 2B).

To examine further the reactivity of these mAbs, we digested purified enzyme with V-8 protease and immunoblotted the digested samples. Among the mAbs tested, two subgroups recognizing different peptide fragments were identified (Figure 3). Thus, mAb hGDH63-10 and hGDH63-11 showed similar bands pattern indicating that these antibodies recognized the same or very similar epitopes. However, hGDH60-6, hGDH60-8, and hGDH91-14 showed different band patterns.

Cross-reactivity of the mAbs with GDH from other mammalian and avian species

In order to examine the cross-reactivities of the mAbs, we isolated the human GDH from the brains of human, cow, pig, dog, cat, rat, and chicken. Total proteins of brain homogenates were separated to SDS-PAGE, transferred on nitrocellulose membranes, and probed with the five hGDH mAbs. The immuno-

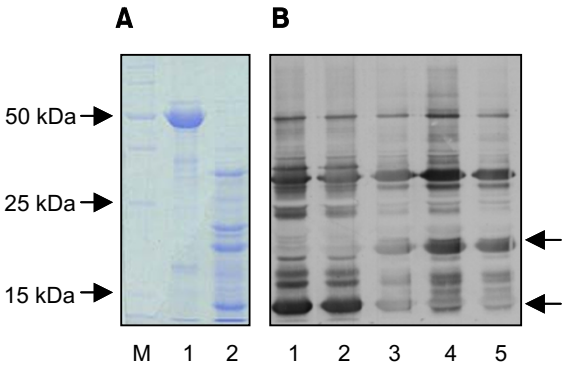

Figure 3. SDS-PAGE of purified human GDH (A) and immunoreactivity of different mAbs with purified human GDH that had been digested with *Staphylococcus aureus* V-8 Protease and separated by SDS-PAGE in a 15% gel (B). Lanes in A is as follows: lane M, marker; lane 1, undigested hGHD; lane 2, digested hGDH. Lanes in B is as follows: lane 1, hGDH63-10; lane 2, hGDH63-11; lane 3, hGDH60-6; lane 4, hGDH60-8; lane 5, hGDH91-14.

reactive bands on Western blot appeared as a single protein band of 55 kDa when hGDH60-6, hGDH60-8, or hGDH91-14 were used as probes (Figure 4A). However, hGDH63-10 and hGDH63-11 showed no immuno-reactivity to any animal band (Figure 4B).

Cross-reactivity of the mAbs with GDH from various rat organs and other animal cell lines

Because the hGDH mAbs recognized GDH in several animals, we examined the immunoreactivities of anti-hGDH mAbs for GDH from various rat tissues, such as that brain and, heart, lung, liver, testes, stomach, muscle. Total protein from the various rat tissues were extracted by the methods described above and immunoblotted with hGDH60-6. As shown in Figure 5A, hGDH60-6 strongly recognized the same protein band on the immunoblots of total proteins of various rat tissues, *i.e.,* brain, heart, liver, stomach and muscle. However, GDH was identified only weakly in the lung and testes.

In order to determine the immunoreactivity of GDH in tumor cell lines with the hGDH60-6, we extracted total proteins from the follwing cell-lines; mouse neuronal cells (PC12), mouse macrophage (J774A1), human hepatocellular carcinoma (HepG2), human squamous carcinoma (SiHa), and human cervical adenocarcinoma (HL3T1) cells, and immunoblotted with hGDH60-6 mAbs. These mAbs strongly recognized a GDH protein band in PC12, J774A1, and HepG2, but weak GDH bands in SiHa and HL3T1 (Figure 5B).

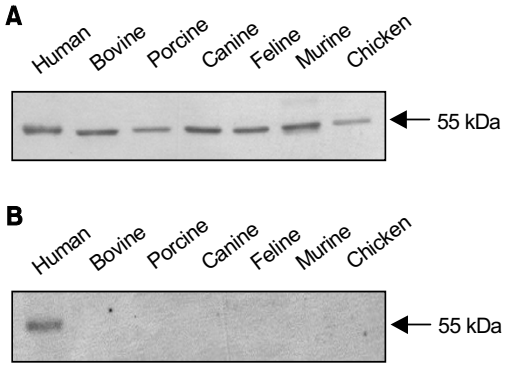

Figure 4. Cross-reactives of human GDH from some mammalian and avian species with mAbs to the human enzyme: lane 1, human; lane 2, bovine; lane 3, dog; lane 4, cat; lane 5, rat; lane 6, chicken; lane 7, pig. Animal brains were removed, and total proteins of the brain homogenates were immunoblotted with the GDH mAbs. (A) An immunoblot probed with mAbs hGDH60-6, hGDH60-8, hGDH91-14. (B) Corresponding immunoblot probed with mAb hGDH63-10, hGDH63-11.

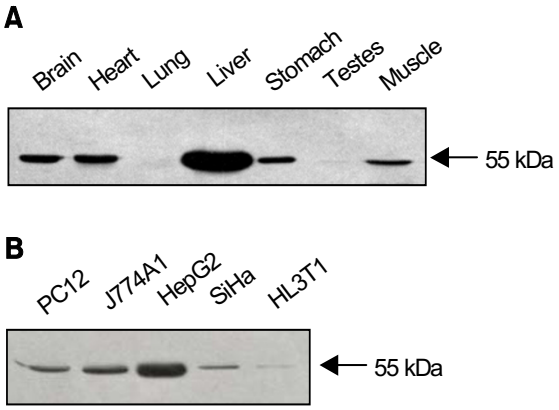

Figure 5. Cross-reactive of GDH from various rat tissues with hGDH mAbs (hGDH60-6) (A): lane 1, liver; lane 2, testis; lane 3, brain; lane 4, heart; lane 5, lung; lane 6, stomach; lane 7, muscle. Total proteins of the rat tissue homogenates were immunoblotted with the hGDH mAb. (B) An immunoblot of total proteins of various mammalians cell lines. Lane 1, mouse pheochromocytoma (adrenal gland) PC12; lane 2, mouse macrophage J774A1; lane 3, human hepatocellular carcinoma (liver) HepG2; lane 4, human squamous cell carcinoma (cervix) SiHa; lane 5, human adenocarcinoma (cervix) HL3T1. The cells were grown in DME containing 10% FBS and total proteins were extracted and immunoblotted.

Discussion

GHD has been studied intensively because of its importance in GDH-dependent neurodegenerative disorders. Much evidence has shown that the enzyme is widely distributed in various tissues, but that its expressional levels and activities vary. Despite exten-

sive biochemical studies on the mechanisms of catalysis, and the fact that a human cDNA encoding the GDH recently has been synthesized and expressed (Yoon *et al*., 2002), little information is available on the structure and regulation of human GDH.

In the present study, we produced a library of mAbs to human GDH. On the basis of the specificities of these mAbs, characterized by Western blot and peptide mapping analysis, they were found to recognize different epitopes of the human GDH enzyme.

It is of interest whether human GDH is immunologically different from other mammalian GDHs. Therefore, we investigated the possibility of using immunoblotting analysis for cross-reactivity testing of anti-human GDH mAbs with various animal brain proteins. Three mAbs (hGDH60-6, hGDH60-8, and hGDH91-14) strongly recognized a protein band of the same molecular weight, equivalent to 55 kDa, in all animals tested, including a human (Figure 4A), whereas hGDH63-10 and hGDH63-11 recognized only human GDH on the immunoblots of mammalian GDHs (Figure 4B). Since only one of the two subgroups of mAbs was found to cross-react with human GDH, human brain enzyme could either differ in terms of amino acid sequences or in protein structure from its counterparts in other animals. It has been suggested that conformational epitopes are more susceptible to denaturing agents, such as SDS, than linear epitopes, and conformational epitopes are usually disrupted during Western blotting (Mole, 1992). Thus, from the immunoblot results, it seems that the human enzyme differs in amino acid sequences rather than in protein structure.

When the inhibition tests were performed on human GDH using the five anti-GDH monoclonal antibodies, two monoclonal antibodies only inhibited human GDH activity (Figure 2B). The maximum extent of this inhibition was 58%. These inhibitory effects of mAb hGDH63-10 or hGDH63-11 on GDH indicate that these mAbs may bind at or near the active site of the enzyme, and thus inhibit the enzyme reaction. Alternatively, the inhibition may be a result of an immobilization of the enzyme brought about by binding of the antibodies to epitopes remote from the active site. Another possibility is that the binding of antibodies causes inactivation by inducing conformational changes. These conformation changes by the enzyme by mAb binding require further investigation and an analysis of the epitope with purified antibody is warranted.

The results of epitope mapping analysis support the possibility that human GDH differs from the other animal GDHs either in terms of amino acid sequences or protein structure (Figure 3). It is, therefore, quite possible that there are differences between human GDH and other animal GDHs in terms of the tertiary or quaternary structures of this epitope, although GDH is known to show high sequence homology among species.

Since the hGDH mAbs recognized GDH in several mammalian brains, we examined the immunoreactivities of the anti-GDH monoclonal antibodies with various rat tissues and tumor cell lines. Immunoblotting showed that GDH in some tissues, such as brain, heart, liver, stomach and muscle, was well expressed at 55 kDa, whereas the GDH in the lung and testes very low levels of expression were observed (Figure 5A). Among the tumor cell lines, GDH was highly recognized in neuronal cells, macrophage and hepatocarcinoma cells; however, the GDH bands in squamous and cervical carcinoma showed at low level of presence (Figure 5B). These results indicated that either GDH expression may be differently regulated in a tissue specific manner or that these differences represent structural differences of the GDH enzyme in various tissues and tumor cell lines.

In conclusion, this study describes the production and characterization of the first complete set of high-affinity monoclonal antibodies against human GDH. On the basis of the specificities of these monoclonal antibodies, characterized by Western blot analysis, inhibition studies, and epitope mapping, we suggest that there are structural differences in the human GDH and other mammalian GDH epitopes. Since antibodies enable the specific and sensitive detection of enzyme in tissues, they could be used for the quantitative evaluation of the enzyme's expression level. Furthermore, the availability of an inexhaustible supply of homogeneous anti-human GDH antibodies could be of great benefit in extensions of the present study, such as immunoaffinity purification, identification of expressed enzymes, and for immunohistochemical study of the brain. In particular, because human GDH mAbs recognized different epitopes of the enzyme, these human GDH mAbs could provide us with detection tools for monitoring therapy in GDH-deficient neurodegenerative disorders.

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