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Tissue Distribution, Molecular Cloning, and Gene Expression of Cytosolic Glutathione Peroxidase in Japanese Monkey

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ABSTRACT—Cytosolic glutathione peroxidase (GPX-1) is an important antioxidant enzyme that scavange hydrogen peroxide in mammalian cells. The level of GPX-1 activity in Japanese monkey (*Macaca fuscata*) tissues was determined and it was found to be high in the liver, kidney, and adrenal gland followed by the small intestine. We also cloned the GPX-1 cDNA that included the whole protein-coding region. The active-site selenocysteine was assumed to be encoded by a TGA codon. Compared to the GPX-1s of other mammalian species, essential residues in catalysis were well conserved in monkey GPX-1. Amino acid substitutions were frequent in the N- and C-terminal regions which are less essential in catalysis. Expression of GPX-1 mRNA was found to be high in the liver, kidney, and adrenal gland, in consistence with the tissue distribution of GPX-1 activity.

Key words: reactive oxygen species, antioxidant enzyme, scavanger, primate

INTRODUCTION

Reactive oxygen species (ROS) are produced as a consequence of aerobic respiration and substrate oxidation. Major ROS are known to be superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$). Since ROS are highly reactive, the occurrence of high levels of ROS may cause metabolic malfunctions and damage to biological macromolecules, resulting in the generating of various diseases including cancer (Matés *et al.*, 1999.), although low levels of ROS are indispensable in many biochemical processes such as the defense system against micro-organisms (Michell, 1984).

Several enzymes are involved in generating ROS (Halliwell and Gutteridge, 1990). We have previously reported that the distribution of ROS generating and scavanging enzymes, including NADH/NADPH oxidase, xanthine oxidase, superoxide dismutase, ascorbate peroxidase, and catalase, were different between tissues of Japanese monkey, *Macaca fuscata* (Fukuhara *et al.*, 2001). Subsequently, Mn- and Cu,Zn-superoxide dismutases have been cloned from various monkey species and they were shown to evolve at variable rates (Fukuhara *et al.*, 2002). These findings suggest the occurrence of tissue- and species-specific systems which generate and scavange ROS in primates.

In the ROS scavanging system, O_2^- is converted to H_2O_2 exclusively by superoxide dismutase and further scavanging of H_2O_2 is catalyzed by few other enzymes. Although two major H_2O_2 scavanging enzymes, namely ascorbate peroxidase and catalase have been examined in our previous study (Fukuhara *et al.*, 2001), another set of important enzymes, namely glutathione peroxidases, remained to be clarified. Glutathione peroxidase (GPX) is a generic name for a family of multiple isozymes that have selenium-dependent glutathione peroxidase activity (Arthur, 2000). There are four GPX isozymes in mammalian tissues; classical or cytosolic GPX (GPX-1; E.C. 1.11.1.9), gastrointestinal GPX (GPX-2), plasma GPX (GPX-3), and phospholipid GPX (GPX-4). These four isozymes show varying tissue distributions and substrate specificities (Brigelius-Flohé, 1999). GPX-1 is the first mammalian selenoprotein to be identified and reduces various hydroperoxides including H_2O_2 (Forstrom *et al.*, 1979; Brigelius-Flohé, 1999). GPX-1 is ubiquitously distributed in various tissues while other GPXs show limited tissue distribution, suggesting that GPX-1 has a major role in protecting tissues from oxidative attack. Therefore, for comprehensive understanding of monkey ROS generating and scavanging systems, we found it appropriate to elucidate the nature of monkey GPX-1.

In the present report, we first examined the tissue distribution of GPX-1 in Japanese monkey. Then, molecular cloning of GPX-1 cDNA was carried out to clarify structural features of monkey GPX-1. The expressional and structural

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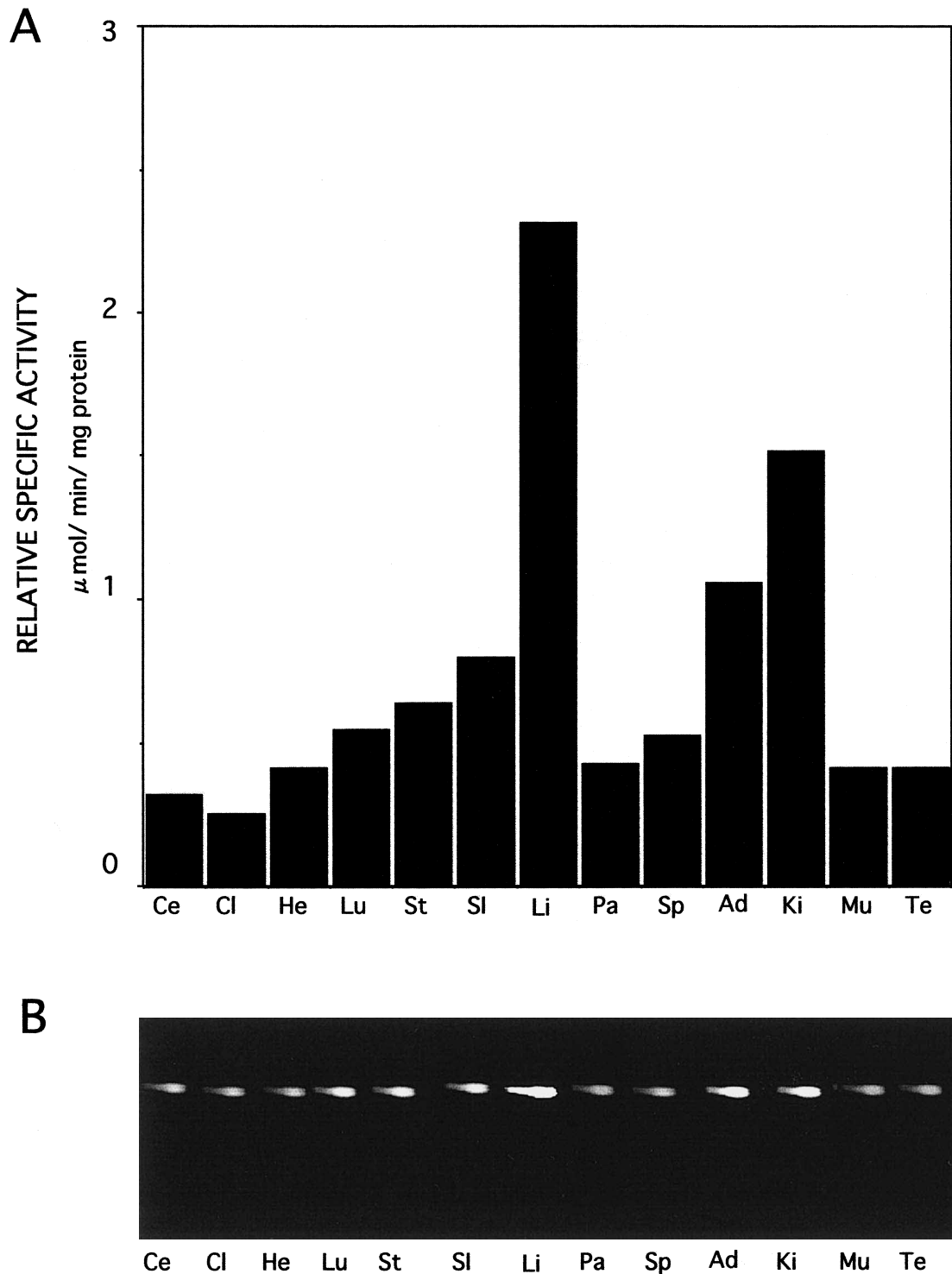


Fig. 1. (A) Relative specific activities of glutathione peroxidase in various tissues of the Japanese monkey. Activity of the enzyme is expressed as μ mol/min under the present assay conditions given in Materials and Methods. Relative specific activity was calculated as the total activity/the amount of the total soluble protein in each tissue. (B) Activity staining of glutathione peroxidase. A portion of the crude homogenate supernatant was subjected to electrophoresis on a 7% polyacrylamide gel in Tris-glycine buffer, pH 8.3. The enzyme activity was stained according to Sun *et al.* (1988) with a slight modification. The upper half of each gel is presented since no activity band was detected in the lower half. Ce, cerebrum; Cl, cerebellum; He, heart; Lu, lung; St, stomach; SI, small intestine; Li, liver; Pa, pancreas; Sp, spleen; Ad, adrenal gland; Ki, kidney; Mu, muscle; Te, testis.

analyses of GPX-1 gene were subsequently achieved by Northern and Southern blotting analyses.

MATERIALS AND METHODS

Chemicals

Reduced glutathione (GSH), glutathione reductase, β -NADPH, 30% hydrogen peroxide, ferric chloride, and potassium ferricyanide were purchased from Wako Pure Ind., Osaka, Japan; Titan One-Tube RT-PCR System and Expand Long Template PCR System were from Roche Molecular Biochemicals, Mannheim, Germany; QIAEX II was from QIAGEN K. K., Tokyo, Japan; the pGEM-T Easy vector was from Promega Corp., Madison, WI; *E. coli* JM109 was from Takara Shuzo Co. Ltd, Otsu, Japan; and Thermo Sequenase cycle sequencing kit was from Amersham, Cleveland, OH. All other chemicals were of reagent grade.

Preparation of monkey tissue homogenate

A young Japanese monkey (*Macaca fuscata*; 6-year-old male)

was used for tissue collection. Cerebrum, cerebellum, heart, lung, stomach, small-intestine, liver, pancreas, spleen, kidney, adrenal gland, muscle, and testis were removed from a monkey immediately after death by exsanguination via bilateral carotid arteries under deep anesthesia with ketamine hydrochloride and sodium pentobarbital, in accordance with guidelines of the Primate Research Institute, Kyoto University. These tissues were stored frozen at -80°C until use. After thawing, tissues were homogenized in 5 volumes of 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1 mM EDTA with a mechanical homogenizer. Each homogenate was centrifuged at $20,000\times g$ for 5 min and the supernatant was used for assaying enzymatic activities and for detecting enzymatic activities after non-denaturing polyacrylamide gel electrophoresis (PAGE).

Assay of enzymatic activities

The total GPX activity was assayed at room temperature, according to Paglia and Valentine (1967). The reaction mixture of 1 ml contained 0.1 M sodium phosphate buffer at pH 7.0, 0.5 mM hydrogen peroxide, 1 mM GSH, 0.1 U of glutathione reductase, 5

1	ATGTGTGCTGCTCGGCTAGCGGCGGGCGGCCAGTCGGTGTACGCCTTCTCGGCGCGC	60
1	M C A A R L A A A A A Q S V Y A F S A R	20
61	CCGCTGGCCGGCGGGAGCCTGTGAGCCTGGGCTCCCTAAGGGCAAGGTGCTACTTATC	120
21	P L A G G E P V S L G S L R G K V L L I	40
121	GAGAATGTGGCGTCCCTCTGA	180
41	E N V A S L SeC G T T V R D Y T Q M N E L	60
181	CAGCGGCGCCTTGGGCCCGGGCCTGGTGGTGTCTCGGCTTCCCGTGCAACCAGTTTGGG	240
61	Q R R L G P R G L V V L G F P C N Q F G	80
241	CATCAGGAGAACGCCAAGAATGAAGAGATTCTGAATTCCTCAAGTACGTCCGACCTGGT	300
81	H Q E N A K N E E I L N S L K Y V R P G	100
301	GGTGGTTTCGAGCCCAACTTCATGCTCTTCGAGAAGTGCGAGGTGAACGGTGCAGGGGCG	360
101	G G F E P N F M L F E K C E V N G A G A	120
361	CACCCTCTTCGCCTTCTGCGGGAGGCCCTGCCAGCCCCAGCGACGATGCCACTGCG	420
121	H P L F A F L R E A L P A P S D D A T A	140
421	CTCATGACCCACCCCAAGCTCATCGCCTGGTCTCCGGTGTGCCGCAACGATGTTGCCTGG	480
141	L M T D P K L I A W S P V C R N D V A W	160
481	AACTTCGAGAAGTTCCTGGTGGGCCCTGACGGTGTGCCCGTACGCAGGTACAGCCGCCG	540
161	N F E K F L V G P D G V P V R R Y S R R	180
541	TTCAGACCATTGACATCGAGCCTGACATCGAAGCCCTGCTGTCTCAAGTCCCAGCAGT	600
181	F Q T I D I E P D I E A L L S Q G P S S	200
601	GCCTAG	
201	A *	

Fig. 2. The nucleotide sequence of cDNA for Japanese monkey GPX-1 mRNA and its deduced amino acid sequence. Box shows TGA codon that is assumed to encode selenocysteine (SeC) of the active site. The data have been submitted to the GenBank/EMBL/DDBJ data bank and are available under the Accession number AB105162.

mM K₂HPO₄, 0.2 mM EDTA, 0.2 mM NaN₃, and 0.1 mM NADPH, and an appropriate amount of tissue homogenate supernatant. In the assay, hydrogen peroxidase was reduced by tissue GPXs with GSH. The oxidized glutathione was then reduced by glutathione reductase with NADPH. The NADPH level was monitored at A₃₄₀ with a model of U-1500 spectrophotometer (Hitachi, Ltd., Tokyo). One unit of GPX activity was defined as the amount of enzyme required for the oxidation of 1 nmol of NADPH /min under the above assay conditions.

Activity Staining of GPX isozymes on non-denaturing PAGE

Electrophoretic separation of GPX isozymes in the tissue homogenate supernatant was carried out using slab gel polyacrylamide method. The gel composition and the buffer system were the same as those described by Ornstein (1964) and Davis (1964). Ferri-cyanide staining procedure for the enzyme activity described by Sun *et al.* (1988) was used with a slight modification. Briefly, the gel was soaked in three changes of 1.5 mM GSH for a total of 45 min, and successively in 1.5 mM GSH containing 0.003% hydrogen per-

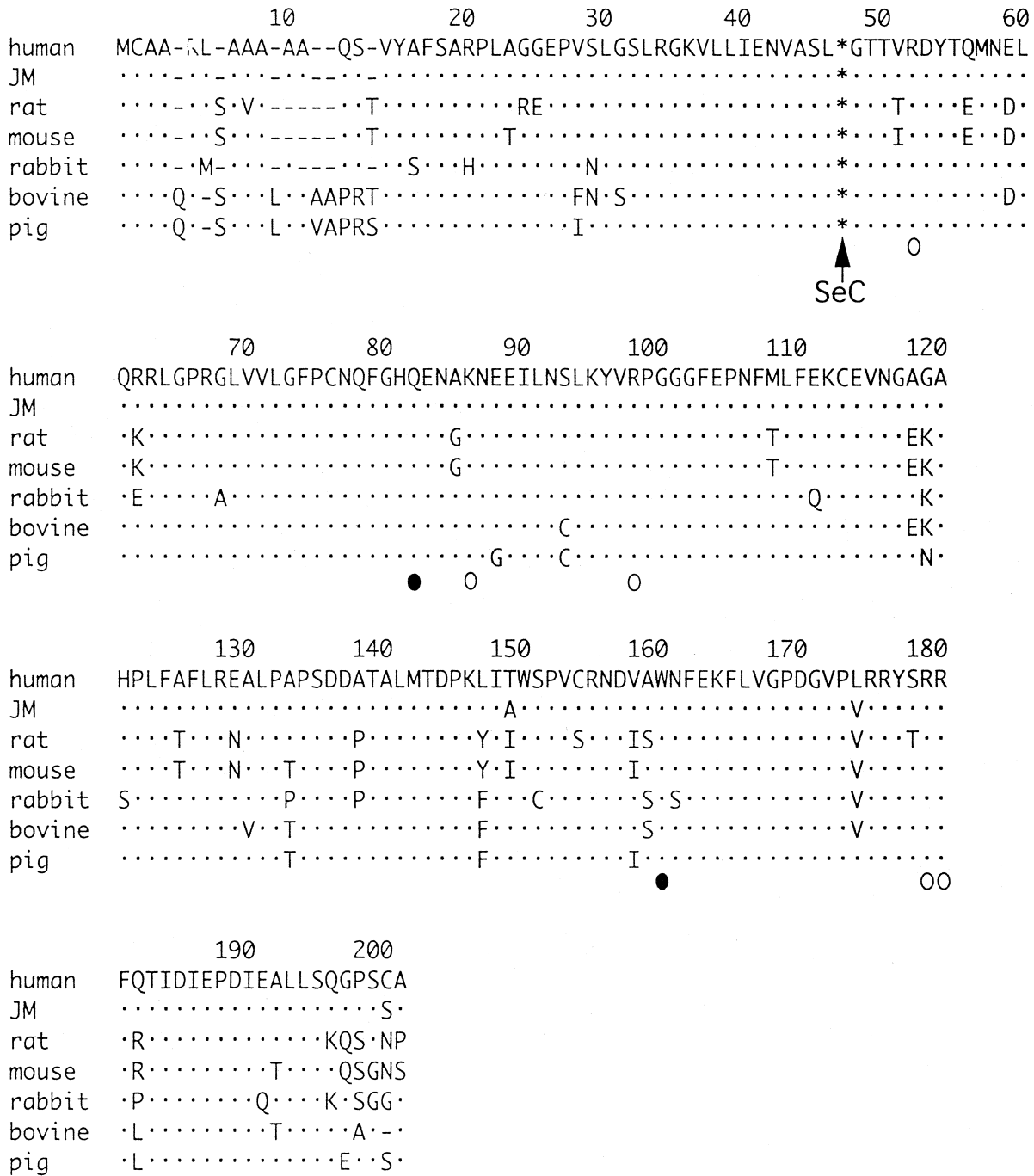


Fig. 3. Comparison of the amino acid sequences of Japanese monkey (JM) GPX-1 (this study; GenBank/EMBL/DDBJ accession No. AB105162) with those of GPX-1s from human (accession No. M21304), rat (M21210), mouse (NM008160), rabbit (X13837), bovine (X13684), and pig (AF532927). The numbering is based on the sequence of human GPX-1. The dots represent the amino acid identity of the reference human enzyme. Dashes indicate insertions/deletions. Asterisk, and open and closed circles show active-center selenocysteine (SeC), and residues being involved in an electrostatic architecture of the way to the active center and in hydrogen bridges for fixing selenium, respectively.

oxide. After a wash with distilled water, the gel was stained with a solution of 0.01% ferric chloride and 0.01% potassium ferricyanide. GPX isozymes were then visualized as a yellow band against dark green background.

PCR cloning of GPX-1 cDNA

The first-round PCR was carried out using the Titan One-Tube RT-PCR System with 0.4 µg total liver mRNA in 25 µl reaction mixture. The primer set was 5'- CCGCTGGCTTCTTGACA and 5'- TTTAGAGGAAACACCCTCAT based upon the cDNA sequence of the respective human enzyme (Chada *et al.*, 1990). The total cycle number of PCR was 30. An aliquot of the PCR products were then used as template for the 30-cycle nested PCR using Expand Long Template PCR System. The primer set was 5'- TCTTGGACAAT-TGCGCCATG and 5'- AAGCAGCCGGGTAGGAG. Nested PCR products were resolved by agarose-gel electrophoresis and the band of cDNAs for GPX-1 was subjected to DNA extraction with QIAEX II. cDNAs were ligated to the pGEM-T Easy vector, used for the transformation of *E. coli* JM109.

Sequences were determined by the dideoxy chain-termination method using a Thermo Sequenase cycle sequencing kit and a DNA sequencer Model LIC-4200L-1 from LI-COR Inc. (Lincoln, NE).

Molecular evolutionary analysis

The nucleotide sequences of mammalian GPX-1 cDNAs were aligned with the aid of DNASIS-Mac 3.0 (Takara Shuzo Co., Otsu, Japan). Phylogenetic analyses on the aligned sequences were per-

formed using the neighbor-joining method of MEGA 2.1 program (Kumar *et al.*, 2001).

Northern blot analysis

Total RNA was extracted from various tissues of the Japanese monkey by the guanidinium thiocyanate-cesium chloride centrifugation method. Fifteen µg aliquots of total RNA were denatured and subjected to electrophoresis in a 1% agarose gel that contained 1.1% formamide. Following the transfer of RNA to a Hybond-N membrane (Amersham Biosci., Little Chalfont, England), the membrane was hybridized in a solution of 6×SSPE, 20% formamide, 2×Denhart's reagent, 0.1% SDS, salmon sperm DNA (final conc., 100 µg/ml), and ³²P-labeled cDNAs for Japanese monkey GPX-1 at 42°C for overnight. The membrane was washed twice in 2×SSPE and 0.1% SDS at 65°C for 20 min, followed by in 0.2×SSPE and 0.1% SDS at 65°C for 20 min.

RESULTS

The level of GPX activity in monkey tissues

The GPX activity was determined in various tissues of the Japanese monkey (Fig. 1A). The relative specific activity of GPX was high in the liver, kidney, adrenal gland followed by the small intestine and stomach. However, it was low in nervous tissues such as cerebrum and cerebellum.

The GPX isozymes were visualized by activity staining

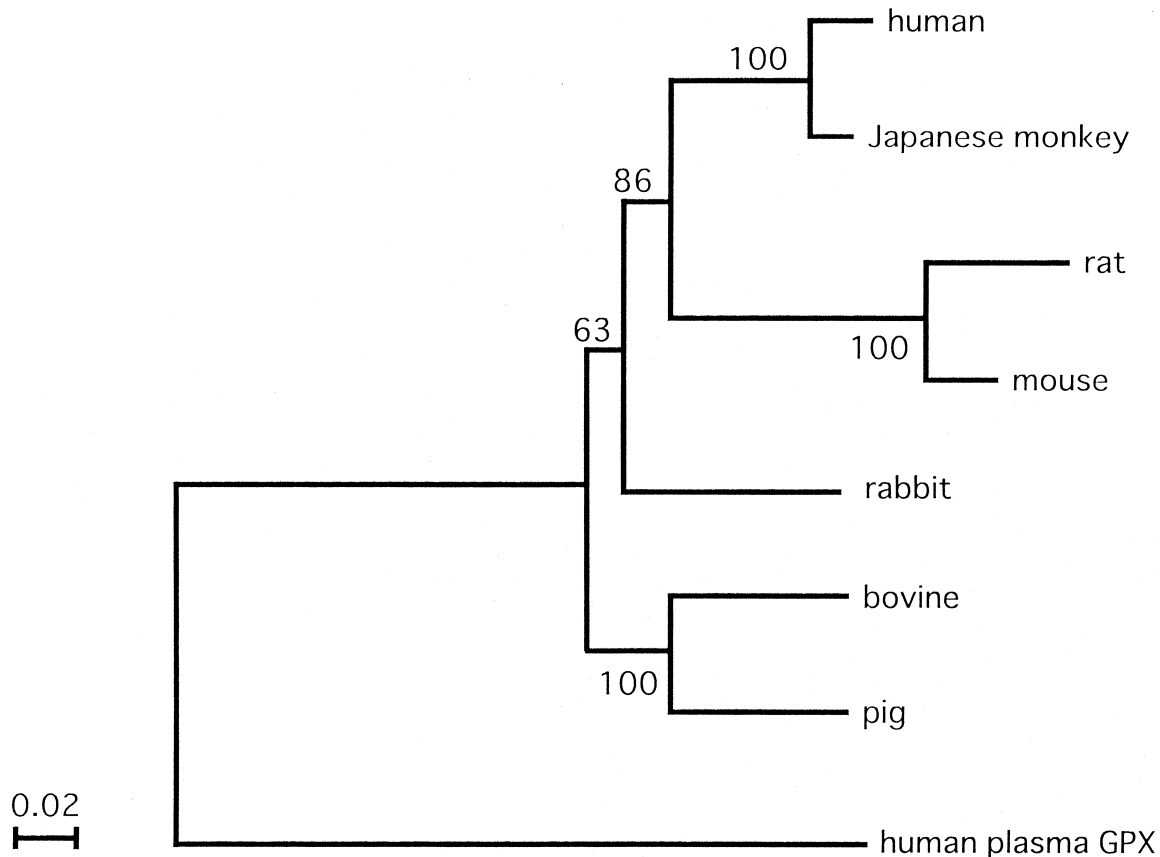


Fig. 4. A neighbor-joining tree of mammalian GPX-1s. Human plasma glutathione peroxidase (GPX-3) was chosen as an outgroup. The scale shown in the lower segment of the tree represents the evolutionary distances, given as the average number of substitutions per site. Bootstrap values (%) obtained with 1000 replicates are shown at each node.

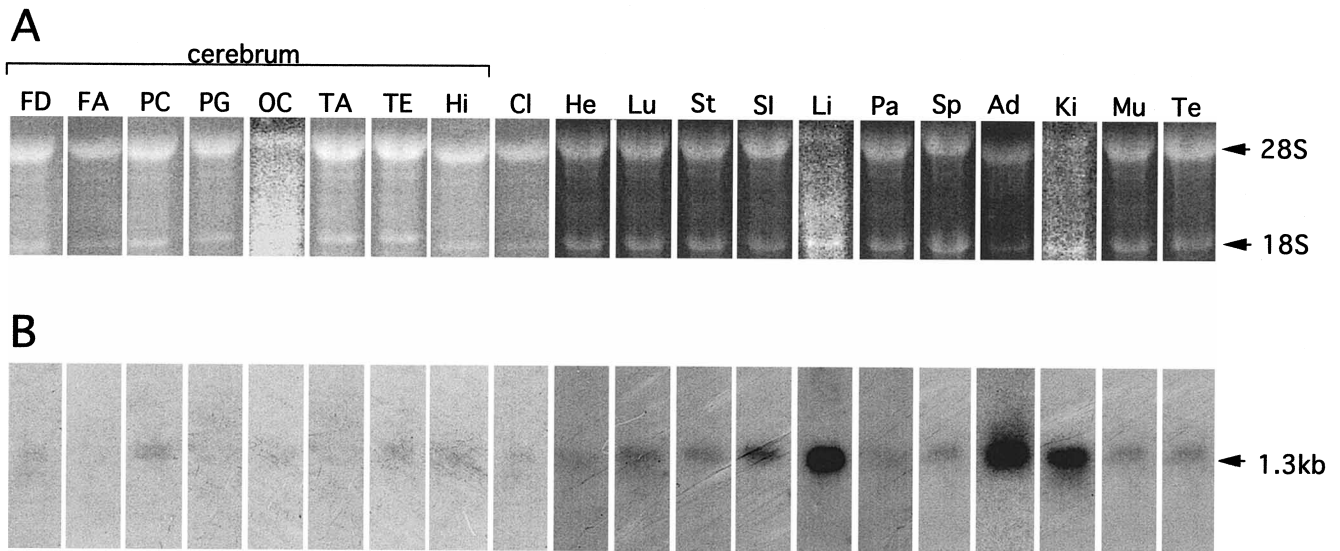


Fig. 5. Northern blot hybridization. (A) Separation of total RNAs by agarose gel electrophoresis. RNA was visualized by staining with ethidium bromide. The amount applied was 15 μ g in each lane. (B) ³²P-labeled cDNA for Japanese monkey GPX-1 was hybridized under high stringency conditions. Used tissues are cerebrum (FD, prefrontal lobe; FA, motor lobe; PC, somatosensory lobe; PG, parietal lobe; OC, occipital lobe; TA, superior temporal lobe; TE, inferior temporal lobe; Hi, hippocampus), Cl, cerebellum; He, heart, Lu, lung; St, stomach; SI, small intestine; Li, liver; Pa, pancreas; Sp, spleen; Ad, adrenal gland; Ki, kidney; Mu, muscle; Te, testis.

on a polyacrylamide gel as shown in Fig. 1B. A single band was detected in all 13 tissues. The color intensity of the band correlated well with the level of GPX activity in each tissue, showing the high color intensity in the liver, kidney, and adrenal gland. The stained band was thought to be the band of GPX-1 considering from the mobility on the non-denaturing PAGE at pH 8.3 since the isoelectric point of GPX-1 was clarified to be 6.16 as shown in the later section. These results showed that the total GPX activity determined by enzymatic procedure in each tissue reflected mainly the activity of GPX-1.

Cloning and structural analyses of GPX-1

The cDNA for Japanese monkey GPX-1 containing the whole protein-coding sequence was cloned. The nucleotide and deduced amino-acid sequences are shown in Fig. 2. A total number of 202 amino acid residues were detected. Fig. 3 shows the deduced amino acid sequence of Japanese monkey with those of other mammalian GPX-1s. Nucleotide sequence similarities to human, rat, mouse, rabbit, bovine, and pig enzymes were 98.5, 84.7, 86.6, 86.3, 88.1, and 91.6%, respectively, and amino acid sequence similarities were 98.5, 84.2, 86.1, 86.6, 88.1, and 92.1%, respectively. The 47th amino acid encoded by TGA was thought to be selenocysteine. Amino acid residues that have been shown to be involved in fixing selenium (Trp160 and Gln82) and directing the substrate toward the catalytic center (Arg52, 98, 179, and 180, and Lys86) (Auman *et al.*, 1997) were conserved in all mammalian GPX-1s. High frequencies of amino acid replacements were obvious in the N- and C-terminal regions. Most of these residues were located at surface of the enzyme, as found by three dimensional

analyses using RasMac ver 2.7 program (data not shown). The pH of the isoelectric-point of Japanese monkey GPX-1 was predicted as 6.16 by the DNASIS-Mac 3.0 program.

Molecular evolutionary analysis

Fig. 4 shows a phylogenetic tree of mammalian GPX-1 constructed from the protein-coding cDNA sequences. As expected, Japanese monkey shows markedly high affinity with human. The most probable relatives to primates were rodents with a high bootstrap value (86%), followed by rabbit and even-toed ungulates. This phylogeny fit well with the recent analysis of 15 nuclear and three mtDNA genes of placental mammals (Murphy *et al.*, 2001).

GPX-1 mRNA levels in Japanese monkey tissues

Expression of gene for GPX-1 in various tissues of Japanese monkey was analyzed by Northern blot hybridization (Fig. 5). GPX-1 mRNA was detected at 1.3 kb. The amount of expression signals was high in the liver, kidney, and adrenal gland, reflecting proportionately the levels of GPX activity levels in tissues. In the brain, there were regional differences in mRNA expression. The expression in somatosensory lobe, inferior temporal lobe, and hippocampus was higher than in other regions.

DISCUSSION

The GPX-1 activity was found to differ in various tissues, being high in the liver, kidney, and adrenal gland followed by the small intestine. Similar variation in the GPX-1 activity have been reported in other mammals such as human (Hornsby and Crivello, 1983; Chu, 1993), rat, and

mouse (Himeno *et al.*, 1993b). These results, including ours, show that GPX-1 is a GPX isozyme with an ubiquitous distribution in various tissues (Brigelius-Flohé, 1999). In the present study, GPX isozymes other than GPX-1 were not detected by activity staining in examined tissues. Although they are known to express in some tissues such as small intestine, the lack of detection might have been due to their low activities or different enzymatic specificities (Sun *et al.*, 1988).

We have previously reported that the distribution of various enzymes involved in producing and scavenging ROS were different between tissues of the Japanese monkey, suggesting the occurrence of tissue-specific ROS-generating and scavenging systems (Fukuhara *et al.*, 2001). The distribution data of GPX-1 in the present study add new information on the monkey ROS-scavenging system. Activity levels of H₂O₂ scavenging enzymes including ascorbate peroxidase, catalase, and GPX-1 have been found to differ significantly among the tissues. This supports our hypothesis relating to ROS-generating and scavenging systems in tissues. While GPX-1 shows important anti-oxidation functions in the liver, kidney, and adrenal gland, similar functions are carried out by ascorbate peroxidase in the small intestine, and by catalase in cerebellum, spleen, small intestine, and heart. Apart from the protection against oxidative attack, GPX-1 may have a role of selenium storage in various tissues, especially in the liver and kidney (Arthur, 2000).

We determined the full-coding nucleotide sequence of monkey GPX-1 cDNA. The 47th amino acid encoded by TGA codon, normally nonsense codon, is thought to be selenocysteine. Forstrom *et al.* (1978) had reported that selenocysteine is an essential residue of the active site of GPX-1. Selenocysteine has also been shown to be encoded by TGA codon in GPX-1s of human (Mullenbach *et al.*, 1987), rat (Yoshimura *et al.*, 1988), and mouse (Chambers *et al.*, 1986), and in a *E. coli* selenoprotein formate dehydrogenase (Zinoni *et al.*, 1987). Several important residues in GPX-1s have been found. First, selenocysteine constituting active site of bovine GPX-1 has been shown to be stabilized by hydrogen bridges provided by the imino group of Trp160 and the amino group of Gln82 (Ladenstein *et al.*, 1986). Secondly, four arginine residues (Arg52, 98, 179, and 180) and a lysine residue (Lys86) provide an electrostatic architecture which directs the donor substrate GSH toward the catalytic center (Aumann *et al.*, 1997). These seven residues are all conserved in mammalian GPX-1s including the Japanese monkey enzyme, showing that essential catalytic properties are similar among the mammalian enzymes.

Successful cloning of GPX-1 cDNA enabled us to examine the expression of GPX-1 gene by Northern analysis. A unique 1.3-kb band detected is probably a transcript of GPX-1 gene, since, although mRNAs of genes for GPX-1, GPX-2, and GPX-4 have been reported to have quite similar sizes, Northern analysis under high stringency conditions has been shown to detect GPX-1 mRNA specifically (Chu *et al.*, 1993). GPX-1 mRNA level appeared high in the

liver, kidney, and adrenal gland followed by the small intestine. The mRNA level in each tissue was directly proportional to the level of GPX activity. The expressional pattern of GPX-1 gene in Japanese monkey appeared similar to those of human (Chambers *et al.*, 1986; Chu, 1993), mouse, and rat (Ho and Howard, 1992; Lei *et al.*, 1995; Munz *et al.*, 1997). However, the GPX-1 mRNA level in guinea pig was reported to be low in the liver, kidney, and heart (Himeno *et al.*, 1993a), showing a distinct species-specificity in expression.

In this study, we have presented the tissue distributions of GPX-1 activity and mRNA levels, and determined the nucleotide sequence of GPX-1 cDNA in Japanese monkey. Since GPX-1 has been shown to play a role of cellular signaling by regulating redox status of the cell (Sandstrom *et al.*, 1994; Krez-Remy *et al.*, 1996; Hampton and Orrenius 1998), our findings on GPX-1 in monkey may contribute significantly to establish a suitable animal model for studying the human diseases related with ROS.

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