Expression of Plasma Glutathione Peroxidase in Human Liver in Addition to Kidney, Heart, Lung, and Breast in Humans and Rodents

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We analyzed the expression of plasma glutathione peroxidase (GSHPx-P) messenger RNA (mRNA) in mouse, rat, and human tissues, using a human GSHPx-P cDNA clone as the probe. Unlike the classical cellular glutathione peroxidase (GSHPx-1), GSHPx-P expression appears to be tissue-specific. In the mouse and rat, kidney expresses an mRNA at a high level detected with the human probe. A signal is also probe. Unlike the classical cellular glutathione peroxidase human tissues, using a human GSHPx-P cDNA clone as the mRNA in midpregnant mice. No signal is detected in cardiac myocytes, mouse lung, epididymis, and the mammary gland of midpregnant mice. No signal is detected in various mouse and rat liver, mouse brain, epididymis, and testis. In human tissues, an mRNA hybridizing to GSHPx-P cDNA is present in liver, as well as kidney, heart, lung, breast, and placenta. We have shown that human kidney expresses a GSHPx-P mRNA, and not a GSHPx-P-like message, by isolating a cDNA clone from a human kidney library in Agt11. From the 412-nucleotide partial sequence of the kidney cDNA, which codes for the 40-170 amino acids of GSHPx-P including the TGA codon for selenocysteine, we found complete sequence identity of the kidney cDNA with GSHPx-P isolated from placenta. The expression of GSHPx-P mRNA in cell lines was also studied. There is some correlation of the expression of GSHPx-P in these cell lines with that in normal tissues. Cell lines that expressed GSHPx-P mRNA or protein included the human hepatocarcinoma HepG2, Hep3B cells, human kidney carcinoma A498 cells, and the human breast cancer SK-BR-3, T47D, MDA-MB-231, and AD/MEC-7 cells. Cell lines that did not express GSHPx-P included human choriocarcinoma BeWo cells, human breast cancer MCF-7, ZR-75-1, and Hs578T cells, and mouse hepatoma Hepa-1 cells.© 1992 by The American Society of Hematology.

MANY FORMS OF ACTIVE oxygen such as hydrogen peroxide, lipid hydroperoxides, superoxide, hydroperoxy and hydroxyl radicals, and singlet oxygen are implicated in human disease. Evidence exists to support a role for oxidant damage in the pathogenesis of rheumatoid arthritis, reperfusion injury, cardiovascular disease, immune injury, and cancer.1-3 Glutathione peroxidase (GSHPx; glutathione-hydrogen-peroxide oxidoreductase, EC 1.11.1.19) has a selenocysteine residue at its active site that catalyzes the reduction of hydrogen peroxide and organic hydroperoxides by oxidizing GSH. Four members of the GSHPx family have been identified in human cells: (1) the classical cellular enzyme, GSHPx-1; (2) the plasma GSHPx, GSHPx-P; (3) the phospholipid hydroperoxide enzyme, PHGPx; and (4) a GSHPx-1-like enzyme, GSHPx-2, expressed in human liver and hepatoma cells.4-6 Additionally, a mouse 24-Kd epididymal secretory protein regulated by androgen has sequence homology with GSHPx, although it is devoid of selenocysteine.7 Based on its epidydimis-specific expression, it appears to be distinct from the other members of GSHPx. Thus, it may be the fifth member in the GSHPx family.8

Yoshimura et al9 detected a messenger RNA (mRNA) that hybridized to a rat GSHPx-P cDNA, and a polypeptide that reacts with a chicken antirat-GSHPx-P antisera on a Western blot isolated from rat kidney. These results strongly suggested that the kidney is the source of the plasma GSHPx activity. However, there are multiple forms of GSHPx that share significant sequence homology across species: the nucleic acid sequence of GSHPx-P has 57% homology with that of cellular glutathione peroxidase (GSHPx-1), and has 67% homology with that of a 24-Kd mouse epididymal secretory protein. It is possible that the signal detected in various tissues is caused by cross-hybridization with yet undetermined members of the GSHPx family. To prove that kidney expresses GSHPx-P we used direct sequencing of a GSHPx-P cDNA isolated from a kidney library. In this study, we showed the sequence identity of the cDNA isolated from a human kidney library to be a GSHPx-P.

We and others have previously reported that the human hepatoma cell lines HepG2 and Hep3B secrete GSHPx-P.4,10 However, Takahashi et al11 failed to detect GSHPx-P mRNA in human liver. Yoshimura et al9 studied GSHPx-P protein expression by Western blotting in 13 rat tissues and found signals in kidney and lung, but not in liver, heart, spleen, thymus, bone marrow, erythrocytes, brain, pituitary and adrenal glands, jejunum, duodenum, or skeletal muscle. These results raised the question of whether there is any correlation between the expression of GSHPx-P in normal tissues and that in cell lines derived from these tissues. Therefore, we have analyzed several normal tissues and cell lines of human, mouse, and rat origin for GSHPx-P expression. We found that human liver, and not mouse or rat liver, expresses GSHPx-P mRNA. Our results are presented in this report.

MATERIALS AND METHODS

Materials. Human cDNA libraries in Agt11 from placenta and kidney were purchased from Clontech Lab (San Diego, CA); GSHPx-P-specific oligonucleotides were synthesized by the Core DNA Synthesis Lab of City of Hope Cancer Research Center (Duarte, CA). Phage Agt11 forward and reverse primers were purchased from New England Biolabs, Inc (Beverly, MA).

Isolation of GSHPx-P cDNA clones. Human stretched placenta and kidney cDNA libraries had 2.3 × 10^9 and 5.0 × 10^9 pfu/mL, respectively, in a 10^9 pfu/mL dilution. We and others have previously reported that the human hepatoma cell lines HepG2 and Hep3B secrete GSHPx-P.4,10 However, Takahashi et al11 failed to detect GSHPx-P mRNA in human liver. Yoshimura et al9 studied GSHPx-P protein expression by Western blotting in 13 rat tissues and found signals in kidney and lung, but not in liver, heart, spleen, thymus, bone marrow, erythrocytes, brain, pituitary and adrenal glands, jejunum, duodenum, or skeletal muscle. These results raised the question of whether there is any correlation between the expression of GSHPx-P in normal tissues and that in cell lines derived from these tissues. Therefore, we have analyzed several normal tissues and cell lines of human, mouse, and rat origin for GSHPx-P expression. We found that human liver, and not mouse or rat liver, expresses GSHPx-P mRNA. Our results are presented in this report.

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respectively. Degenerate oligonucleotides were made according to our previously described GSHPx-P peptide fragments. They were used as probes to isolate GSHPx-P cDNA clone from a placenta library. Specific oligonucleotides were made after we sequenced the placenta GSHPx-P clone. These oligonucleotides were used to screen for GSHPx-P clones in a human kidney library. Screening, phage plating, and transfer to nitrocellulose filters (Schleicher & Schuell Inc, Keene, NH) were performed in duplicate as described. Secondary and tertiary screening were performed after isolating the positive plaques to ensure that single clones were obtained in each isolate.

**DNA sequencing.** We completely sequenced our placenta GSHPx-P cDNA clone bidirectionally by subcloning the insert of the Xgt11 clone into pBluescript (pBS; Stratagene, San Diego, CA). For sequencing reactions, either Sequenase (US Biochemical, Cleveland, OH) or linear polymerase chain reaction (PCR) sequencing was used. Ig Suite from the Core DNA facility of the Cancer Center, City of Hope (IntelliGenetics, Inc, Mountain View, CA) was used to analyze and manage the sequencing data.

The kidney GSHPx-P cDNA clone was sequenced in Xgt11 directly. The insert was amplified with Xgt11 forward and reverse primers by PCR. The amplified fragment was isolated from the unincorporated primers by precipitation with 20% polyethylene glycol in 2 N NaCl. It was then sequenced directly by the linear PCR sequencing method.

**Northern analysis.** Total RNAs were isolated from human and animal tissues or cell lines. Tissue sources and numbers of successful isolation are summarized as follows.

1. Mouse tissues: liver: 1X from a Balb/c, 1X from each of a δ and a δ Swiss Webster, and 1X from a δ BM-1 strains; kidney: 3X from δ Balb/c, 1X from each of a δ and a δ Swiss Webster, and 1X from a δ Swiss CD-1 strains; heart: 1X from each of a δ and a δ Swiss Webster, and 4X from δ Swiss CD-1 strains; lung: 1X from a δ Swiss CD-1 strain; mammary gland: 2X from 14 day pregnant δ Swiss Webster strain; epididymis: 1X from a δ BM-1, and 1X from a δ Swiss Webster strain; uterus: 1X from a δ Balb/c strain; brain and testis: 1X from a δ Swiss Webster strain.

2. Rat tissues were all isolated from Sprague-Dawley rats (Charles River Lab, Wilmington, MA), including liver: 2X; kidney cortex and medulla: 2X each; heart: 1X; cardiac myocytes: 2X.

3. Human tissues: liver: 3X normal tissue; kidney: 3X normal tissue; heart: 1X normal tissue; lung: 3X normal and 1X tumor tissue; breast: 4X normal tissue.

Isolation of cardiac myocytes from Sprague-Dawley rats was performed following a previously described procedure with minor modifications. After the heart was perfused on a Langendorff apparatus with Ca²⁺-free Tyrode's solution, myocytes were obtained by perfusing the heart with 50 mL of recirculating Tyrode's solution containing 50 mg of collagenase B (Boehringer-Mannheim Biochemicals, Indianapolis, IN), 5 mg protease (type XIV; Sigma) for 45 minutes at 37°C.

Total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction method. Ten micrograms of RNA was resolved by formaldehyde-containing gels. Random primed human and chicken mRNA was used as probes. The hybridizing and washing conditions were described previously.

**Cell lines and ²⁵Se-labeling.** Human cell lines used included hepatoma HepG2 and Hep3B cells, kidney carcinoma A498 cells, breast carcinoma MCF-7, SK-BR-3, T47D, MDA-MB-231, ZR-75-1, HS578T cells, and choriocarcinoma BeWo cells (American Type Culture Collection, Rockville, MD). An adr-expressing variant of MCF-7 cells, AdrMCFC-7, was kindly provided by Kenneth Cowan (National Cancer Institute, Bethesda, MD). Mouse hepatoma Hepa-1 cells were kindly provided by Oliver Hankinson (University of California, Los Angeles, CA). A GSHPx-1 transfectant of MCF-7 cells, MCF-7H6, was established as described. At least two independent isolations of mRNA were performed on each of these cell lines.

BeWo cells were cultured in Kaighn's nutrient mixture F-12 (Irvine Scientific, Santa Ana, CA) supplemented with 15% fetal bovine serum. The other cells were grown in either Dulbecco's modified Eagle's medium (DMEM)/F12 or Richter's improved MEM zinc ion medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum. Selenoproteins were labeled by incubating cells in growth media supplemented with 100 nmol/L ⁷⁵Se-selenious acid for 4 to 6 days before harvesting.

**Protein analysis and enzyme assays.** Immunoprecipitation of ⁷⁵Se-GSHPx-1 and ⁷⁵Se-GSHPx-P was performed with rabbit antihuman—RBC—GSHPx-1 antisera and antihuman—GSHPx-P antisera, as previously described. The immunoprecipitates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**RESULTS**

We screened 60,000 plaques and isolated eight GSHPx-P clones from a human placenta clone library both cell lines. We found a cDNA clone that includes the full coding sequence, and monomeric phospholipid hydroperoxide glutathione peroxidase, and expressed only in human liver and hepatoma cells (manuscript in preparation); PxP is the porcine monomeric phospholipid hydroperoxide glutathione peroxidase (PHGPx)⁷; Px2 is a tetrameric and catalytically active GSHPx expressed only in human liver and hepatoma cells⁸ (manuscript in preparation); Px1 is the classical human cellular GSHPx-1; and Ep1 is a 24-Kd mouse epididymal secretory protein regulated by testosterone.⁹ We have recently determined the nucleotide sequence of 315 bp of the coding region of a human PHGPx cDNA. The deduced amino acid sequence of this portion has 95% homology with porcine PHGPX (unpublished data).

First, we studied the expression of GSHPx-P mRNA in mouse tissues and mouse hepatoma Hepa-1 cells using a human GSHPx-P cDNA as the probe. As shown in lanes 1 to 8 of Fig 2A, and lanes 9 of Fig 2C, kidney expresses a signal. Similar to mouse liver, Hepa-1, a mouse hepatoma cell line, does not express any mRNA that hybridized with the human GSHPx-P probe. Figure 2B shows the mRNA signals hybridized with the chicken β-actin probe that are used as a control for the quality and quantity of the mRNA preparation. The weak signal at higher molecular weight is at the same location as the abundant 28S rRNA that often hybridizes with the probes. The 18S rRNA has a molecular weight similar to β-actin. Because actins are composed of a number of isoforms highly conserved in evolution, the β-actin probe appears to hybridize with α-skeletal and
α-cardiac actin mRNAs that are coexpressed at high levels in striated muscles, in addition to two α-smooth muscle actin mRNAs present in sarcomeric muscles. This result in double bands in some of the lanes in the β-actin hybridized blots. To confirm the results obtained with rodent tissues, we also analyzed several human tissues for GSHPx-P expression. Multiple human samples were shown in lanes 5 through 8 of Fig 2C and Fig 3A. With the results in double bands in some of the lanes in the p-actin where ZR-75-1, Hs578T, and MCF-7 do not. Other human RNAs of different tissues are isolated from different individuals. These data show that human liver, as well as kidney, heart, lung, and breast, express GSHPx-P mRNA.

Because Yoshimura et al. did not detect any signal from rat heart by Western analysis, we wanted to know if the signal that we detected in mouse heart was specific to mice. We have examined the protein secreted by several human cell lines to study the correlation of GSHPx-P expression in vitro versus in vivo. As shown in Fig 2C, lanes 10 through 14, and lanes 3 and 5 of Fig 2E, four of seven human breast cell lines express an mRNA hybridized to GSHPx-P cDNA. SK-BR-3, T47D, MDA-MB-231, and ADR-MCF-7 have a detectable message, whereas ZR-75-1, Hs578T, and MCF-7 do not. Other human cell lines analyzed for GSHPx-P mRNA expression are shown in Fig 2E. These include hepatoma HepG2 (lane 1), kidney carcinoma, A498 (lane 2), and choriocarcinoma, BeWo (lane 5), cell lines. HepG2 and A498 cells express GSHPx-P mRNA, whereas BeWo cells do not have a detectable mRNA.

We have examined the protein secreted by several human cell lines to determine whether the mRNA expressed by these cells, which hybridized with the GSHPx-P probe, codes for GSHPx-P. As shown in Fig 4, A498 (lanes 1 through 3), ADR-MCF-7 (lanes 4 through 6), MCF-7H6 (lanes 7 through 9 and 13 through 15; a GSHPx-1 transfected cell line), and HepG2 cells (lanes 10 through 12) were labeled metabolically in media containing 100 nmol/L [35S]selenocysteine for 4 to 6 days and the [35S]sulfenate proteins were analyzed by SDS-PAGE. Figure 4 shows that A498, ADR-MCF-7, and HepG2, but not MCF-7H6, secrete
Fig 2. Northern analysis of GSHPx-P mRNA in human and animal tissues, and human cell lines. (A, C and E) show a 1.6-kb mRNA hybridized to a human GSHPx-P cDNA probe; and (B, D, and F) show a 1.9-kb mRNA hybridized to an β-actin probe. (A and B) show the same set of mouse RNAs isolated from Hepa-1 (lane 1), kidney (lane 2), liver (lane 3), testis (lane 4), epididymis (lane 5), uterus (lane 6), heart (lane 7), and brain (lane 8). (C and D) are the same set of RNAs isolated from rat kidney cortex (lane 1), rat kidney medulla (lane 2), rat heart (lane 3), rat cardiac myocytes (lane 4), human heart (lane 5), human lung (lane 6), human breast (lanes 7 and 8), the mammary glands of a pregnant mouse (lane 9), and five human breast cancer lines (lanes 10 through 14). The breast cell lines are SK-BR-3 (lane 10), T47D (lane 11), MDA-MB-231 (lane 12), ZR-75-1 (lane 13), and Hs578T (lane 14). (E and F) are the same set of RNAs isolated from other human cell lines, including HepG2 (lane 1), A498 (lane 2), AdrMCF-7 (lane 3), BeWo (lane 4), and MCF-7 (lane 5).

Fig 3. Northern analysis of mRNA isolated from human tissue. (A) mRNA hybridized to GSHPx-P cDNA. (B) mRNA hybridized to β-actin. Duplicate samples were from two individuals. Lanes 1 and 2 are from normal kidney, lanes 3 and 4 are from normal liver, lane 5 is from normal heart, lanes 6 and 7 are from normal lung, and lane 8 is from tumor lung of the same individual as lane 7.
PLASMA GLUTATHIONE PEROXIDASE (GSHPx-P)

Fig 4. $^{75}$Se-labeled proteins obtained from four human cell lines resolved by SDS-PAGE. Lanes 1 through 9, 11, and 12 are selenoprotein immunoprecipitates recovered in the conditioned media, whereas lanes 13 through 15 are cytosolic selenoproteins that are controls to show the specificity of the antisera. Lanes 1, 4, 7, and 13 are immunoprecipitates with preimmune sera; lanes 2, 5, 8, 11, and 14 are immunoprecipitates with anti-RBC–GSHPx-1 antisera; lanes 3, 6, 9, 12, and 15 are immunoprecipitates with anti–GSHPx-P antisera; and lane 10 is the pattern of total cytosolic selenoproteins in HepG2 cells.

a 22.5-Kd selenoprotein that is immunoprecipitated with antihuman GSHPx-P antisera.

**DISCUSSION**

Yoshimura et al.° using immunoblotting and Northern hybridization techniques, identified that rat kidney and lung, but not rat liver or heart, expressed GSHPx-P in 13 tissues analyzed. These data suggest that kidney is the source of plasma glutathione peroxidase. However, as shown in Fig 1, there are apparently five isozymes in the glutathione peroxidase family. To prove that the signal detected in kidney by GSHPx-P probes is that of GSHPx-P per se, a nucleic acid or an amino acid sequence analysis of a kidney GSHPx-P cDNA or protein is necessary. Our partial sequence of a cDNA clone isolated from a human kidney cDNA library has shown its identity.

When the deduced amino acid sequence of five potential isozymes in the glutathione peroxidase family are compared, there is higher homology between human GSHPx-P and mouse epididymal 24-Kd protein than between human GSHPx-P and human cellular GSHPx-1. When the nucleic acid sequences at the coding region are compared, human GSHPx-P has 67% homology with mouse epididymal 24-Kd protein and 57% homology with human GSHPx-1. However, the 24-Kd epididymal secretory protein is not likely to be the mouse GSHPx-P, based on the facts that (1) the cDNA of the 24-Kd epididymal protein did not hybridize with any mRNA isolated from kidney and other nonepididymal tissue,° (2) the amino acid homology of human and rat GSHPx-P is 90%, whereas that of human GSHPx-P and mouse 24-Kd epididymal protein is 71%,° and (3) the cDNA coding for the 24-Kd protein does not appear to contain a TGA codon.°

In addition to the mouse kidney, positive signals were also detected in mRNA isolated from mouse epididymis, heart, lung, and mammary gland. It is likely that the signal detected in epididymis is caused by cross-hybridization of the mRNA of the 24-Kd epididymal secretory protein with a GSHPx-P cDNA probe. However, based on the specificity of the probe, which was shown in the isolation of the human kidney GSHPx-P cDNA, it is more likely that these other tissues are expressing GSHPx-P mRNA. Additionally, the HepG2, A498, and Adr′MCF-7 cell lines that expressed an mRNA that hybridized to GSHPx-P cDNA also secrete a selenoprotein that can be immunoprecipitated with antihuman GSHPx-P antisera. This evidence supports the conclusion that these tissues express a GSHPx-P mRNA.

We have detected GSHPx-P mRNA in human liver, kidney, heart, lung, and breast. We are confident in these results because, with the exception of human heart, multiple samples were analyzed. Takahashi et al.°° have reported that human liver does not express GSHPx-P mRNA, but no data was shown, and they isolated GSHPx-P clones from a human fetal liver cDNA library.°° However, because GSHPx-P is not expressed in mouse and rat liver, there is a definite species difference in its expression in liver. Additionally, unlike rodent kidney, human kidney does not have overwhelming levels of GSHPx-P mRNA. Considering the mass of liver, the total GSHPx-P expressed in human liver may be equivalent to that expressed in kidney.

We have also detected a signal in mRNA isolated from rat heart, rat cardiac myocytes, mouse heart, and human heart; mouse lung and human lung; mouse breast and human breast. Our result indicates that there may be no species difference in GSHPx-P expression in heart, lung, and breast. However, GSHPx-P is expressed at a very low level, if any, in one human tumorous lung sample. More samples need to be analyzed to determine whether there is a general pattern of reduced GSHPx-P mRNA level in tumorous human tissues.

We have also studied the expression of GSHPx-P mRNA or protein in 12 cell lines, and found a fair correlation with
the tissues from which they were derived. HepG2 and Hep3B, human hepatoma cell lines, are similar to human liver and express GSHPx-P; Hepa-1, a mouse hepatoma cell line similar to mouse liver, does not. A498, a human kidney carcinoma cell line, expresses a GSHPx-P mRNA and protein as does human kidney; human breast cancer cells SK-BR-3, T47D, MDA-MD-231, and Adr’/MCF-7 are similar to human breast, and express GSHPx-P. However, other breast cancer cells, ZR-75-1, Hs578T, and MCF-7, and BeWo, a human choriocarcinoma cell line, are unlike their tissues of origin, and do not express GSHPx-P.

Although the physiologic role of GSHPx-P has not been established, its expression in heart, lung, and breast tissues, in addition to kidney and human liver, suggest that the antioxidant activity may be important in providing local extracellular protection, in addition to providing a potential oxidant scavenger role in the plasma. This local protection maybe particularly important in humans, because human plasma has several-fold lower levels of GSHPx activity compared with rodent plasma (unpublished data). The differential expression of GSHPx-P in breast cancer cell lines is interesting. Because the normal mouse breast tissues were obtained from pregnant females, and the normal human breast were most likely obtained from postpregnant females, its expression in this tissue may be developmentally regulated.

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