



Human Hepatocytes: An Untapped Resource for Functional Genomic Studies of Cytochrome P450 Enzymes

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ABSTRACT

To examine the activity, genotype, and expression of cytochrome P450 enzymes in cryopreserved human hepatocytes and assess the potential of this resource for functional genomic studies of human enzymes, hepatocytes from 51 different human donors were evaluated for activity of the CYP1A2, CYP2A6, CYP2C9, CYP2C19 and CYP2D6 enzymes with standard probe drugs. DNA from a single vial for each human cryopreserved hepatocyte sample was isolated and tested for 6 alleles of CYP2A6, 4 alleles of CYP2C9, 3 alleles of CYP2C19 and 15 alleles of CYP2D6. Preliminary data suggested some extreme enzyme activities are predictable based on the expressed alleles of each gene. However, the genotype and enzyme phenotype did not match in all instances. This suggests additional allele tests and/or sequencing of the responsible gene and further investigation of the mechanism responsible may uncover new and novel alleles in some of these human genes. For CYP2C19 enzyme activity there was the lack of any relationship between enzyme activity and genotype. Even the distribution of CYP2C19 enzyme activities in the hepatocytes as a group was very different from that of the other cytochrome P450 enzyme activities. The metabolism of some drugs by human hepatocytes may be affected by processes other than hepatic enzyme activity.

INTRODUCTION

Since the completion of the human genome project the next great challenge rests with the efficient discovery of functional gene variations in the human genome. This discovery would be facilitated if it was possible to evaluate multiple genes rather than single genes, and also if the mechanism(s) by which new gene variations elicit their functional effects could be addressed. The purpose of this research was to determine if cryopreserved human hepatocytes could be used for functional genomic research of metabolic enzymes.

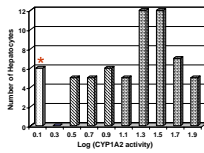
METHODS

Human hepatocytes: 51 lots of different cryopreserved human hepatocytes previously evaluated for CYP1A2, CYP2A6, CYP2C9, CYP2C19 and CYP2D6 enzyme activities with standard probe drugs (CYP1A2 - phenacetin O-deethylase; CYP2A6 - coumatetralil 7-hydroxylase; CYP2C9 - tolbutamide 4-hydroxylase; CYP2C19 - mephenytoin 4'-hydroxylase; CYP2D6 - dextromethorphan O-demethylase). Repeated assessment of enzyme activities in hepatocytes that were cryopreserved for up to 2 years had previously established their long-term viability.

Genetic testing: Human cryopreserved hepatocytes were rapidly thawed at 37°C and diluted into 49 mL of thawing buffer provided by In Vitro Technologies, Inc. The thawed hepatocytes were centrifuged at 2-3,000xg and the thawing buffer removed. The hepatocytes were immediately processed and DNA isolated with a Qiagen DNA mini kit for DNA tissue extraction according to the manufacturer's instructions. Typically, 150-200 µL of DNA was recovered from a single vial of human hepatocytes (2.5 x10⁶ cells; 5,000-15,000 DNA copies per µL). The isolated genomic DNA was subsequently tested for CYP2A6 alleles *1x, *2, *3, *4, *5, *9; CYP2C9 alleles *2, *3, *5 and *6; CYP2C19 *2, *3, *4 and CYP2D6 *1x, *2x, *4x, *5, *4, *5, *6, *7, *9, *10, *17, *30, *35, *41 and *43.

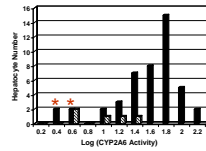
Western blots: Additional hepatocytes were obtained from In Vitro Technologies, Inc. for the purpose of assessing cytochrome P450 enzyme expression. Supernates containing specific expressed cytochrome P450 enzymes served as reference (CYP2A6, CYP2C9, CYP2C19 and CYP2D6) and immunassay kits for the same cytochrome P450 enzymes (Western immunoblotting kits) were purchased from BD-Bioscience (San Jose, CA). Hepatocytes were homogenized in ice-cold Tris buffer (100 mM, pH 7.5) with 0.05 mM EDTA and centrifuged at 35,000g for 30 minutes and the supernatant re-centrifuged at 105,000g for 1 hr. The microsomal pellet was suspended in 100 µL Tris buffer and protein content determined by the Coomassie protein assay. A fixed microsomal protein amount was subjected to electrophoresis on a 12% SDS-PAGE gel for 2 hrs at 120 V. The proteins were transferred by electroblotting to nitrocellulose, the nitrocellulose was then blocked with 2% powdered nonfat milk and probed with immuno-specific antibodies for the individual cytochrome P450 enzymes according to the manufacturer's instructions.

Distribution of CYP1A2 enzymatic activity in human hepatocytes samples



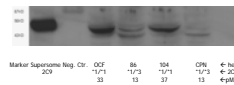
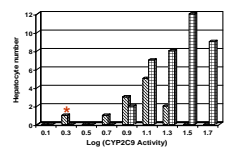
Phenacetin O-deethylase activity in 51 human hepatocytes samples. The population log(CYP1A2 activity) appears to distribute into 2 groups of hepatocytes with little or no CYP1A2 activity (stippled bar, asterisk) shown at the far left. The remaining hepatocytes are shifted to higher activities, but the distribution does not appear unimodal. Genetic testing was not carried out on these samples due to limited information on functional CYP1A2 variations.

Distribution of CYP2A6 enzymatic activity



The distribution in activity from 51 human hepatocytes that were also genotyped for CYP2A6*2, *3, *4, *5 and *9 alleles. Cross-hatched bars represent hepatocytes samples with genotypes of *9/*9, *2/*1, *2/*9, *1/*5 (anticipated to express diminished CYP2A6 activity), while samples with a *9/*1 or *1/*1 are shown in the black solid bars. The hepatocytes population appears to distribute into 2 groups, with hepatocytes with diminished activity falling toward the low end and hepatocytes with high CYP2A6 activity expressing 2 apparently normal copies of the CYP2A6 gene. Discrepancies between the CYP2A6 activity and genotype (e.g., those samples in solid black marked with asterisk at left of diagram) are candidates for CYP2A6 gene cloning and sequencing.

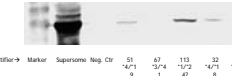
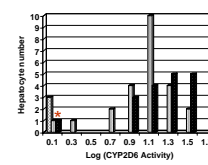
CYP2C9 activity, genotype and protein expression level



A) CYP2C9 activity and CYP2C9 genotypes for 51 human hepatocytes. Cross-hatched bars represent hepatocytes with *1/*3, *1/*5, *3/*2 or *2/*2 genotypes (assumed to express lower CYP2C9 enzyme activity, and which are predictably shifted to the lower end of the enzyme activity spectrum). The enzyme distribution histogram seems to indicate the presence of one hepatocyte sample, indicated with an asterisk (*), CYP2C9 *1/*5 that appears different from the rest.

B) Western blot of CYP2C9 enzymes. The lower enzyme activity associated with the *1/*3 genotype was also associated with a lower level of CYP2C9 expression. This suggests the *3 allele is not only associated with lower affinity for its substrates, but possibly also a lower level of enzyme stability or gene expression.

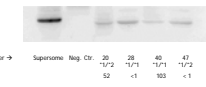
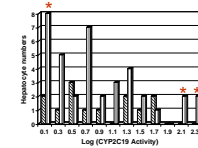
CYP2D6 activity, genotype, and protein expression level



A) Distribution of 51 human hepatocytes based on their CYP2D6 activity and CYP2D6 genotype. Cross-hatched bars are hepatocytes with no functional genes for CYP2D6 (*3/*4 or *4/*4) genotypes. Gray bars represent hepatocytes with at least one inactive gene (*4/*1, *4/*2, *3/*1, etc., or two alleles with diminished activity (*41/*10, *10/*10) and the remaining represent hepatocytes with two functional CYP2D6 alleles (*1/*2, *35) or one functional and one allele with somewhat diminished activity (*1/*10, *2/*41, *35/*9, etc.). The hepatocytes clustered at the left end of the graph appear to exhibit lower than anticipated activity, and one (marked with asterisk CYP2D6 *1/*10 genotype) is a candidate for sequencing and evaluation.

B) Western blot of CYP2D6 enzyme level. The activity and CYP2D6 genotype appear to match quite closely in the western blot for this enzyme. The deficiency in CYP2D6 expression is associated with no detectable enzyme (*3/*4, *4/*4), intermediate activity (*4/*1) is associated with lower activity, and normal expression of the CYP2D6 enzyme (*1/*2) is related with higher CYP2D6 enzyme expression.

CYP2C19 activity, genotype, and protein expression level



A) Distribution of 51 human hepatocytes based on their CYP2C19 activity and genotype. Histogram of CYP2C19 activity and genotype shows a bimodal distribution, but the majority of samples display extremely low CYP2C19 activity, with only 4 samples exhibiting very high CYP2C19 activity. The CYP2C19*2 allele was the only inactive allele detected and samples with a *1/*2 genotype are shown in the cross hatched bars. Hepatocytes with a CYP2C19*1/*1 genotype are given by the bars with a solid line in the middle. Samples marked with an asterisk displayed the same genotype but markedly different activities of CYP2C19 and would be candidates for further study.

B) Western blot of CYP2C19 enzyme level. Evaluation of the CYP2C19 enzyme amount in some hepatocytes did not explain this marked difference in enzyme activity. Twenty micrograms of microsomal protein were used for each of the samples, but only 5 µg were employed for the supernates expressing CYP2C19 and negative controls.

RESULTS

- Plots of hepatocyte CYP1A2, CYP2A6, CYP2C9, CYP2C19 and CYP2D6 activity showed a distribution for each enzyme activity that contain outliers.
- Immunoblots indicated that low CYP2A6, CYP2C9 and CYP2D6 activity typically correlated with a corresponding low expression of each enzyme.
- A CYP2C9*5/*1 genotype, a CYP2D6*1/*10 genotype and two CYP2A6 *1/*1 genotypes were associated with extremely low activity of the corresponding enzymes and might be worthwhile to clone and sequence.
- CYP2C19 activity was extremely variable in many of the hepatocyte samples expressing apparently identical CYP2C19 genotypes and containing measurable CYP2C19 enzyme. This suggests that additional uncharacterized alleles or poorly defined processes were occurring that affect CYP2C19 activity.

DISCUSSION

Although in many cases enzyme activity and expression are correlated and consistent, with each P450 analysis there are hepatocyte samples that reveal a lack of correlation between activity and genotype or expression. Specifically, there appear to be samples in which a normal activity genotype is expressed but is associated with inactive enzyme, or lower than expected activity. This presents an opportunity to utilize that sample to clone and characterize novel human allelic variants of the gene under study. Simultaneous RNA analysis would allow the rapid and facile determination of whether these expression differences are transcriptional or post-transcriptional events.

CONCLUSIONS

These results indicate that combining hepatocyte genotype and phenotype information could help direct efforts to identify new functional variants in cytochrome P450 genes. Relating enzyme activities, levels and genotypes may facilitate efforts to define the primary mechanism responsible for human variation in drug metabolism. The evaluation of many enzymes and genes simultaneously and the ability to assess the mechanism responsible for differences in enzyme activity has significant promise and potential for human functional genomic research.