Molecular Genetics of the Human Cytochrome P450-Dependent Monooxygenases

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Imperial Cancer Research Fund, Molecular Pharmacology Group, Edinburgh, UK, and *Imperial Cancer Research Fund, Human Genetic Resources Laboratory, Potters Bar Herts, UK, †University of Edinburgh, Department of Pathology, Edinburgh, UK

WOLF, C.R., SMITH, C.A.D., SMITH, G., GOUGH, A.C., BRYANT, S., SPURR, N.K. and HARRISON, D.J. Molecular Genetics of the Human Cytochrome P450-Dependent Monooxygenases. Tohoku J. Exp. Med., 1992, 168 (2), 73-81 —— In this work, the role of genetic as well as environmental factors in determining cytochrome P450 isozyme levels in man have been studied. Simple DNA based assays for the identification of individuals nulled at the CYP2D6 locus are described and have been applied to investigate whether this gene defect is associated with altered cancer susceptibility. In contrast to literature reports, in no cancer type were poor metabolizers underrepresented, indeed in several cancers the mutant allele frequency was increased. A model using human tumours grown as xenografts is described that should help elucidate the factors which regulate P450 levels in man. —— human cytochrome P-450; cancer susceptibility; mutant allele frequency; CYP2D6; human tumor xenograft

Cytochrome P450-dependent monooxygenases play a central role in the metabolism of drugs, chemical toxins and carcinogens. Individuality in the expression of P450 genes may therefore play an important role in individual susceptibility to diseases such as cancer. In this report we describe work on the environmental and genetic factors which regulate cytochrome P450 levels in man.

The reactions catalyzed by these enzymes usually involves the incorporation of an atom of molecular oxygen into the substrate. In most cases this results in increased hydrophilicity and facilitates excretion. However, in certain cases this metabolic process results in the activation of the prodrug or procarcinogen converting it into its ultimate carcinogenic form. Possibly as a consequence of the central role of this enzyme system in detoxification, the cytochrome P450-dependent monooxygenases have evolved into a series of multigene families and in man there may be fifty or more distinct enzymes active in foreign compound metabolism (Nebert et al. 1991). Each of these forms exhibits its own unique

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range of substrate specificity.

As a result of the central role of the P450s in carcinogen metabolism it is expected that cellular differences in P450 isozyme content will be a factor in individual susceptibility to cancer (Wolf 1990). It is therefore of central importance to understand the tissue distribution of specific cytochrome P450 isozymes, and the factors which are involved in their regulation.

In many ways the cytochrome P450 system can be viewed as an adaptive response system to environmental challenge. This conclusion is based on the observation that exposure to a particular environmental chemical will induce hepatic levels of a cytochrome P450 isozyme active in its metabolism. It is now well known that a host of factors, both exogenous as well as endogenous, can influence P450 levels. Most of the studies on this theme have been carried out in experimental animals and current information on the regulation of specific P450 isozymes in man is sparse.

In addition to the transient regulation of cytochrome P450s by foreign compounds and hormones it is also known that certain P450 forms in man exhibit genetic polymorphism (Forrester and Wolf 1990). Individuality resulting from such polymorphisms in certain cases is of central importance in determining drug pharmacokinetics and as a consequence drug side-effects. Individuality of this nature may therefore also be an important factor in susceptibility to environmentally-linked diseases such as cancer.

Of particular interest in this regard is a genetic polymorphism at the CYP2D6 gene locus where nulled individuals have a severely compromised ability to metabolize the marker drug debrisoquine and approximately thirty other drugs (Eichelbaum 1988). There are also several reports indicating that this polymorphism is also linked to altered susceptibility to a wide variety of cancers, including cancers of the lung, breast, bladder, liver and kidney. Some of these reports have proved to be equivocal, as a consequence of the limitations of the pharmacokinetic assay used to determine CYP2D6 phenotype. In order to clarify this issue we decided to develop a DNA based assay which would allow unequivocal determination of individuals carrying mutations at this locus. In this report we describe the assays we have developed and present data on the association of CYP2D6 genotypes with susceptibility to a range of different cancer types. In addition, we describe the development of a model which can be applied to understand the factors which determine the expression and regulation of specific cytochrome P450 isozymes in human tissues.

**Materials and Methods**

All chemicals were obtained from the usual commercial sources and were of the highest purity available.

Human liver samples were obtained from organ transplant donors and were frozen in liquid nitrogen within 1 hr of removal from the patient. Patient information has been
described previously (Forrester et al. 1992). Human colon and breast xenografts were derived from solid tumour samples obtained at surgery. Certain of the mice bearing human tumours as xenografts were treated with a variety of cytochrome P450 inducing agents including 1,4-bis [2-(3, 5-dichloropyridyloxy) benzene] (TCPOBOP). (Single injection of 3 mg/kg); phenobarbital (80 mg/kg x 3); dexamethasone (100 mg/kg x 4); 3-methylcholanthrene (100 mg/kg x 4); 1,6-naphthoflavone (80 mg/kg); Aroclor 1254 (500 mg/kg, single injection) and clofibric acid (200 mg/kg x 4). All injections were intraperitoneal in saline or corn oil and at 24 hr intervals before use with the exception of TCPOBOP and Aroclor 1254 which were single injections 3 or 7 days before use, respectively. Blood samples from either control or cancer patients taken for identification of CYP2D6 genotype were obtained from hospitals in Scotland, Sheffield, London or Oxford. All individuals are Caucasians. Control samples were obtained from random blood samples from Clinical Chemistry Departments in Sheffield or Edinburgh or from volunteers. Tissue microsomal and cytosolic fractions were obtained by differential centrifugation. mRNA was isolated by the guanidinium hydrochloride method.

Western blot analysis was carried out on microsomal or cytosolic proteins (15 μg) following their separation on either 9% or 12% SDS polyacrylamide gels, for P450s and GSTs, respectively. Specific cytochrome P450 or glutathione S-transferase isozymes were then identified using polyclonal antisera raised against purified rat or human P450 or GST enzymes. The specificity of the antibodies and the proteins which they recognize has been described previously (Forrester et al. 1992). Immunohistochemical localization of cytochrome P450 isozymes in the tumour xenografts was performed on formalin fixed tissues using a biotin/avidin peroxidase detection system as described by Harrison et al. (1989). Antibodies were used at a dilution of 1: 50. Northern blot analysis was carried out as described by Meehan et al. (1988). mRNA was separated on 1% denaturing agarose formaldehyde gels and transferred overnight to Hybond N membranes. Membranes were then probed with cDNA probes to human P450s and reprobed with a β actin cDNA to ensure equal loading. Hybridizations were carried out at 65°C in 2× SSC 0.1% SDS and 0.1% sodium pyrophosphate. Genotyping for normal and mutant CYP2D6 alleles was carried out using the polymerase chain reaction on whole blood or isolated DNA samples using the methods we have described previously (Gough et al. 1990; Wolf et al. 1990; Spurr and Wolf 1991).

Results and Discussion

Hormonal, environmental, as well as genetic factors are known to determine tissue cytochrome P450 levels. The best characterized genetic polymorphism in the human P450 system is associated with the CYP2D6 gene locus. It is clear however, that in addition to the genetic polymorphism other factors are important in determining the level of CYP2D6 protein (Gonzalez et al. 1988). Fig. 1 shows that in the panel of liver samples we have studied the level of CYP2D6 protein is subject to considerable variation (16-fold). This variation is so large that it is likely that heterozygotes carrying one normal and one mutant CYP2D6 allele will have overlapping phenotypes with homozygotes with two normal alleles. It is now clear that in the majority of poor metabolizers no CYP2D6 protein is produced. This is exemplified by individual seven in Fig. 1 who was shown by genotyping to be homozygous for the frame shifting mutation at the junction of intron 3 and exon 4 (Gough et al. 1990).

In order to study the association between the CYP2D6 polymorphism and
Fig. 1. Individuality in the expression of CYP2D6 debrisoquine hydroxylase P450. a) Western blot analysis; b) Relative band intensity from a panel of liver samples.

Fig. 2. Diagrammatic representation of three PCR assays used to identify mutant CYP2D6 alleles. Alleles a, b and c are the G→A transition, gene deletion and A deletion in exon 5, respectively. Horizontal arrows indicate the position of the PCR primers; vertical arrows indicate the site of digestion with diagnostic restriction enzymes. In assay 2; i and ii indicate the two independent PCR assays which are required.
cancer susceptibility, simple DNA based genotyping assays have been developed. The assays we have developed are shown in Fig. 2.

Our assays analyze for three gene inactivating mutations; these are: the frame shifting G to A mutation at the junction of intron 3 and exon 4 (allele frequency in PM's of 0.8) [allele a] (Gough et al. 1990; Kagimoto et al. 1990; Hanioka et al. 1990); a gene deletion (allele frequency in PM's of approximately 0.12); [allele b] Gough et al. 1990; Gaedigk et al. 1991) and a rare base pair deletion in exon 5 (allele c) (allele frequency in PMs is approximately 0.02; Kagimoto et al. 1990). In the original assay we described to identify PMs (Gough et al. 1990) we analyzed the common G to A mutation by taking advantage of the fact that the mutation removed a BstNI restriction site. Amplification of the CYP2D6 gene over the site of this mutation using the polymerase chain reaction (PCR) generated a product that did, or did not (mutant), digest with BstNI (Fig. 2a). This assay is approximately 80% predictive of phenotype identifying individuals homozygous for allele 'a' and those heterozygous for allele 'a' and allele 'b' i.e., \((0.8 + 0.12)^2 = 0.85\), i.e., 85% of PMs. In a modified version of this assay (Wolf et al. 1990) the mutation in exon 5 was also analyzed using a mismatched oligonucleotide PCR primer which introduced an MspI (HpaII) restriction site at the point of this mutation. This assay, which involves two PCR reactions, identifies individuals carrying all three mutant alleles described above apart from those homozygous for the gene deletion, where no PCR product is obtained (Wolf et al. 1990). In a further refinement of the assay a mismatched oligonucleotide was used which introduced a DraIII restriction site at the site of the exon 5 mutation (Spurr and Wolf 1991; Gough et al. in preparation). In addition an oligonucleotide pair was introduced which specifically amplified the pseudogene CYP2D8. Using this strategy all the above mutations could be identified in a single PCR reaction. The methodology for the standard assay we currently use which involves two PCR reactions has recently been published (Smith et al. 1992).

These assays were then applied to study different cancer populations (summarized in Fig. 3) (Wolf et al. 1992). Of particular note was that the proportion of PMs or mutant alleles in the lung cancer population was almost identical to that found in controls indicating that this polymorphism is not a factor in susceptibility to this disease. This was also the case for carcinoma of the breast. Interestingly, in a variety of other cancer types, the mutant allele frequency, and in certain cases the proportion of PMs, was increased. This was particularly apparent in melanoma, bladder cancer and leukaemia. In the latter case it is interesting that blast cells were taken for the analysis and therefore the increase in PMs could be due to allele loss in the region of chromosome 22 where CYP2D6 maps. Recent studies have shown that this gene locus maps to within 4.2 cM of the platelet derived growth factor B chain gene whose oncogenic homologue is the sis oncogene (Gough et al. 1993).
Regulation of human cytochrome P450s

The loss of cytochrome P450 expression and inducibility in cells in culture makes it difficult to determine the factors which regulate human P450 forms. In order to establish a model to study human P450 regulation we have determined cytochrome P450 isozyme expression in human tumours and also whether the levels of the forms detected can be regulated by exogenous agents. Human colon and breast tumours were grown as xenografts in immune deprived mice and the ability of a variety of foreign compounds to regulate cytochrome P450 expression determined (Fig. 4). Cytochrome P450 expression in both tumour types in the absence of inducing agent was extremely low but detectable and was equivalent to tumor tissue obtained directly from patients. However, significant induction in the levels of a variety of human P450's could be observed following the administration of a P450 inducing agent. Immunohistochemical analysis demonstrated that the induction was within the tumour and Northern blot analysis

Fig. 3. Frequency of poor metabolizers (A) and mutant CYP2D6 alleles (B) in cancer patients relative to controls (horizontal lines). Values in brackets represents the number of patients studied.
confirmed that in the case of CYP2B, a human P450 not a murine P450 had been induced. Both \( \beta \)-naphthoflavone (\( \beta \)-NF) and 3-methylcholanthrene (3-MC) induced the expression of a CYP1A protein (not shown), presumably CYP1A. Interestingly 3-MC also induced the expression of members of the CYP2A and CYP4A gene families (not shown). Phenobarbital, dexamethasone and most interestingly TCPOBOP could all induce the expression of a CYP2B protein in the colon xenograft (Fig. 4). TCPOBOP even at a dose of 75 \( \mu \)g per animal was
an effective inducer. It appears that this compound, as well as dexamethasone, which exhibit profound species differences in their ability to induce CYP2B, will induce CYP2B in man. Similar to the observations in mice, TCPOBOP was also an effective inducer of P450s from other gene families including CYP2C and CYP3A. These experiments demonstrate that human tumours express a number of cytochrome P450 isozymes and that the expression can be regulated by a variety of exogenous agents. The use of tumour xenografts will provide a valuable tool for understanding the factors which determine cytochrome P450 isozyme levels in man.

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