COMMENTARY

Developments and perspectives on the role of cytochrome P450s in chemical carcinogenesis

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Introduction

Cytochrome P450-dependent monoxygenases (P450s) are a supergene family of enzymes that catalyse the oxidation of lipophilic chemicals through the insertion of one atom of molecular oxygen into the substrate. They are responsible for the metabolism of a wide range of endogenous and foreign compounds, and in man the transformation of many drugs and chemical carcinogens is mediated by the P450 system (1). It is paradoxical that P450s are involved both in the detoxification of many compounds and also, in some cases, production of the ultimate carcinogen.

The major challenge of P450 research is to understand the role that human P450 forms play both separately and together in chemical carcinogenesis, and to determine the relative contribution of genetic and environmental factors in an individual’s capacity to metabolize and activate carcinogens. Realization of these goals may identify genes that are important in individual susceptibility to cancer. The purpose of this Commentary is to highlight some of the approaches used in addressing these problems, to discuss both their value and shortcomings, and to suggest possible future advances. Clearly P450s are not the only determinants in chemical carcinogenesis, and other xenobiotic uptake and metabolizing enzymes and DNA repair systems are of considerable importance; many of the approaches adopted to study the P450 system have been or can be applied to these other systems.

This Commentary is a personal, and therefore biased view of the subject and does not aim to be any sort of comprehensive review. There are many excellent reviews of the biochemistry and molecular biology of P450s to which the reader is referred (1-4), and for the uninitiated reader the nomenclature system of Nebert et al. (5) is essential in understanding the relatedness and diversity of P450s. Although there is still considerable room for improvement, and there have been significant changes to the nomenclature since its inception, the adoption of this system has at least allowed P450s identified in different laboratories to be compared.

Some of the approaches that can be applied to study the function of human P450s are shown in Table 1.

Biochemical and immunological approaches

It is difficult to purify human P450s, not least because of problems obtaining suitable liver samples. In view of these difficulties there have been, and are likely to remain, relatively few studies using a biochemical approach. However, antisera directed against purified human or rodent P450s, which cross-react with human P450s in microsome preparations, have provided important tools for investigating P450 function in vitro. In essence, these antisera can be used to determine the relative content of various P450 forms in human liver microsomes by immunoblotting, and then the amount of cross-reacting material is correlated with the samples’ known enzymic activities or ability to convert a specific compound to a mutagen (as judged by direct assay of the product or in the Ames test). This approach relies on there being a sufficiently wide range of expression of the relevant P450s in the human liver samples available; indeed significant inter-individual variation of P450s is a striking feature in man. A further extension is to show that the relevant antiserum can inhibit the particular activity, and that antisera towards other P450 forms do not inhibit. If a wide panel of antisera are available, then this approach has the advantage that there is no preconception as to which P450 form(s) will be involved in a particular activity. It has been successfully used to identify the human P450 forms involved in the activation of aflatoxin B1 (6,7) and a wide range of other P450 substrates. The general agreement in the data obtained between different laboratories using this strategy validates this approach.

In view of the relatedness of P450s, particularly those within family 2, it has been important to establish that antisera used are at least subfamily specific. This problem has been solved using extracts from cells that have been genetically engineered to express a single human P450 from a cDNA clone. However, as we shall see, certain P450s are so closely related that anti-protein antisera may never distinguish them. Antisera towards synthetic peptides of the most divergent regions of related P450s have been used successfully in distinguishing these P450 forms and represent an important way forward in this research area (8).

Molecular genetic approaches

Purified P450s from rats and mice have provided the probes (both antibody and oligonucleotide) for isolating their corresponding cDNAs, and in turn the homologous human cDNAs and genes. This approach has a major advantage in that minor P450s from almost any tissue can be studied. Single human P450 forms can now be expressed, using recombinant DNA techniques, in yeast, mammalian cells or vaccinia expression systems, and even in very high quantities in insect cells. This allows the substrate specificity of individual human P450 forms to be determined directly, and the testing of metabolites from specific chemical- plus P450 pairs in the Ames test. Alternatively, stable expression of individual P450s in suitable yeast strains or mammalian cell lines, and probably in the near future bacterial cells, permits direct mutagenicity testing. The application of these approaches represents a major advance in understanding the P450 system, and using this strategy it will eventually be possible to generate cells expressing combinations of P450s so that their relative role in carcinogen activation as well as deactivation can be established.
Table I. Advantages and limitations of different approaches to the study of the function of human P450s

<table>
<thead>
<tr>
<th>Biochemical approach</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Isolation of proteins</td>
<td>isolation of several isozymes in functional form simultaneously provides antigens for antibody production</td>
<td>poor availability of tissue only abundant forms can be isolated isolation of novel forms in most extrahepatic tissues not possible using current technology</td>
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<td>Use of antibodies as inhibitors or to quantify isoenzyme expression</td>
<td>allows relative isoenzyme content in a panel of samples to be established allows the relative contribution of individual forms in catalysing a reaction to be established</td>
<td>unless human standards available only relative isoenzyme content can be established specificity of antibodies needs to be confirmed (peptide antibodies may circumvent this problem)</td>
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| Recombinant DNA approach (i.e. expression of human proteins from their cDNAs in vivo) | cDNA expression in heterologous systems establishes the capacity of an enzyme to catalyse a specific reaction for mutation and toxicity tests the reactive metabolites are produced within the target cell allows generation of antibodies to human proteins using fusion proteins (e.g. β-galactosidase—P450) site-directed mutagenesis allows structure—activity relationships to be determined | does not clarify the role of the isozyme in carcinogenesis in vivo comparisons are complicated by the difficulty in establishing the amount of functional recombinant protein produced allelic variants of the expressed cDNAs may have different specificities properties of the host cell used for expression may influence the activities measured, e.g. reductase limitation? presence of cytochrome b5? |

| Genetic models | Use of recombinant inbred or congenic mouse lines role of a P450 gene family in a pharmacological or toxicological response can be unequivocally assigned | limited to the identification of gene cluster rather than specific genes at present limited to studies in mice using transgenic mammals, background activities could be a complicating factor labour intensive carcinogenicity cannot be used as end point |
| Expression of human P450s in transgenic animals | in vivo model; can be used in pharmacokinetic modelling toxicity and carcinogenicity testing certain advantages to using transgenic Drosophila, e.g. ease of genetic manipulation. Background P450 activities low and can be deleted. Relatively easy to generate complex combinations of human carcinogen-metabolizing enzymes quickly using tissue-specific promoters the P450 can be expressed in a specific tissue. This approach also allows the properties of the carcinogen itself to be studied, i.e. ability to migrate from site of activation to a target cell | time consuming and technically difficult for multigene families of homologous proteins at present limited to the study of mouse, therefore extrapolation to man may be difficult |
| Gene deletions | allows the role of an enzyme in chemical toxicity and carcinogenesis to be unequivocally assigned | |

It is important to note that many of the above techniques give different types of information and it is their collective application that will yield the information needed.

There is still extremely little information on this latter theme.

On the face of it, these approaches appear to be the ultimate way to assess the potential contribution of individual P450 forms to chemical carcinogenesis. However, there are several drawbacks. Firstly, the nature of the target cell used for the P450 expression may influence the activity observed, i.e. the nature of the cell membranes, the level of P450 reductase, the presence of cytochrome b5 which appears to activate some P450s and inhibit the function of others. Also, if mutation is an end point, other factors, such as DNA repair, may be rate limiting in determining mutation frequency. This would mask any catalytic differences between P450 forms in chemical activation. Secondly, a problem arises because of the overlapping substrate specificity between P450 forms. The importance of a P450 in catalysing a reaction is often based on a comparative assessment. In transfected cells the relative levels of functional protein derived from a cDNA may be considerably different. Indeed, in transiently transfected cells this will be the case for the same
expressed cDNA from experiment to experiment. It is therefore only possible to compare activities of different P450 forms if the level of active protein is determined. Thirdly, a problem that will be true whenever cloned human cDNAs are used is that of genetic (allelic) variation (e.g. point mutations); therefore the cDNA being tested may have properties distinct from other allelic forms of the same protein.

For an enzyme system such as the P450s where many of the genes are not essential for life, a significant level of genetic polymorphism may be expected. A study of restriction fragment length polymorphisms (RFLPs) and sequence analysis indicates that this is the case for the P450 system. Many of the polymorphic sites affect the coding sequence and change the encoded amino acid. Although there may be only one amino acid substitution between allelic variants, this may be sufficient to modify significantly the substrate specificity. The ability of one amino acid to make such a change has been elegantly demonstrated in the mouse CYP2A subfamily where the Phe209Leu substitution in P450c9 is sufficient to convert it from a coumarin 7-hydroxylase to testosterone 15α-hydroxylase (9). Therefore the interpretation of experiments using cloned human cDNAs must take into account the possibility that different alleles express P450s with different substrate specificity and/or turnover number. At present there is insufficient information to be able to predict which polymorphic amino acid substitutions are likely to influence substrate specificity. Modelling of P450c9 to the known crystal structure of P450cam gives no real clues as to why the Phe209Leu substitution in P450c9 is critical in determining substrate specificity. Until we have a better model for mammalian P450s, or a clearer understanding of what the determinants of substrate specificity are, allelic variation must be a serious consideration in interpreting these expression experiments.

The diversity of P450s in mammals seems to be controlled at the level of single genes coding for single proteins, and not by gene rearrangements as for immunoglobulins. However, there have been sufficient reports of alternative-splicing in P450 genes to warrant consideration of its significance. Aberrant splicing of mRNAs has been observed in the human, rat and murine CYP2B, CYP2C, CYP2D gene families and in the rat and murine CYP4A gene family. Several types of alternative splicing have been observed, leading to mRNA products that have missing exons, replaced exons or insertions; the open reading frame in the mRNA may be disrupted leading to the potential translation of a truncated polypeptide, or alternatively a polypeptide with an insertion (see e.g. 10,11). In many cases the potential polypeptide products would not be capable of binding haem, as the amino acid residues required would be missing. Although protein products corresponding to these alternative mRNAs have not always been detected, it is possible that they may have some function in their own right or a subtle role in influencing the P450 system. For example, as they retain the N-terminal hydrophobic tail, they may modulate membrane function or possibly substrate binding. The process of alternative (aberrant) splicing often appears to occur concomitantly, and in a sense competes with, the generation of normal transcripts (see 11). This may be an important factor in determining the level of functional P450 produced.

Genetic approaches

Although allelic variations leading to amino acid substitutions have the potential to explain genetic differences in drug and carcinogen oxidation, this does not appear to be the case for the best-understood case, human debrisoquine hydroxylase deficiency. Rather, there appear to be three different mutations that account for the majority of the mutant alleles associated with this defect (12-14). Debrisoquine hydroxylase is a member of the CYP2D subfamily of genes which contain nine exons and eight introns. The most frequent mutant allele [frequency in poor metabolizers (PM) ~ 80%] is a G to A transition at the junction of intron 3 and exon 4, which modifies the splice site and leads to the loss of the first base of exon 4 in the MRNA with a resulting frameshift mutation. A base pair deletion in exon 5 (allele frequency ~ 5-10%) also leads to a frameshift. The third mutation is a gene deletion (allele frequency ~ 10%). In all cases no functional protein is produced. It is not inconceivable, however, that there are other alleles at this gene locus which result in amino acid substitutions and alter substrate specificity. Such alleles could explain the lack of correlation in debrisoquine oxidation rates between homozygous normal and heterozygotes. There are of course other possible explanations for this observation.

Assays based on the polymerase chain reaction (PCR) are now available which are 90% predictive of the metabolizer phenotype (12-15). These simple assays use a DNA sample from blood, mouthwash or other tissue sources. The correlation between metabolizer phenotype and susceptibility to lung, bladder and other smoking-related cancers has long been of significant interest but the data obtained using phenotyping assays have proved controversial. The use of the PCR assay will now allow unequivocal identification of genotype, and the screening of large populations will allow any statistically significant correlations to be ascertained.

There is a problem in reconciling the correlation of the PM phenotype with reduced susceptibility to smoking-induced cancer, namely that debrisoquine hydroxylase does not seem to activate any compounds in cigarette smoke to mutagenic forms. Should this gene locus (CYP2D) prove to be associated with cancer susceptibility it is conceivable that this could be due to linkage with another cancer-causing gene on chromosome 22q rather than a direct involvement in the cancer process.

Of course more than one genetically determined component is likely to be involved in the susceptibility to a potential carcinogen, and in this regard any study using human populations is complicated by their heterogeneity. As an experimental system in understanding multigenic phenomena, the mouse has many advantages. Genes encoding xenobiotic-metabolizing enzymes have been mapped, e.g. not just P450s but also glutathione S-transferases, UDP-glucuronosyl transferases, epoxide hydrolase, etc. (see 16). There are many genetically determined differences in drug-metabolizing enzyme activity between different strains of inbred mice. Inbred mice strains, however, are in themselves genetically pure, so there is no problem associated with allelic variation. The use of mouse genetics represents a powerful tool for delineating pathways of carcinogen metabolism and has already been used to great effect in the study of the association of the Ah locus with cancer susceptibility (17). On the other hand, there has only been extremely little use of mouse genetics to understand the function of the P450 system. This will undoubtedly change in the future, e.g. the use of recombinant congenic strains will allow the possible identification of genetic combinations that contribute to chemical carcinogenesis. [Each recombinant congenic strain carries a small fraction of the genome of one strain on the genetic background of the second strain, thus non-linked genes controlling the same trait become separated in different strains and can be studied individually (18).]

The generation of congenic mouse lines is difficult and such approaches are likely to be superseded by more sophisticated...
new technologies in molecular genetics. Firstly, transgenic mice could be made which express single human P450s in a tissue-specific manner. The effect of such heterologous expression on susceptibility to potential carcinogens in the short and long term could be assessed in comparison with the wild-type mouse. Alternatively, manipulation of embryonic stem cells could produce mice that lack completely, or are hemizygous for a single P450 gene, and the effect of these genetic modifications on susceptibility could be assessed. Although these types of experiments can address the question of the role of individual P450s, they cannot overcome the problem associated with extrapolation from mouse to man. The use of transgenic animals expressing human P450 genes will undoubtedly be extensively applied in future years. There are alternatives to mammals for these studies that are currently being developed. For example, mammalian P450s and glutathione S-transferases have been successively expressed in *Drosophila* (19). The power of *Drosophila* genetics and the relatively low background activities make this a potentially exciting model for development. A current goal for the application of molecular genetic approaches in understanding carcinogenesis is to express many of the human enzymes involved in carcinogen metabolism simultaneously in the same cell or organism. For organisms such as yeast and *Drosophila* this could be quite easily achieved, for others, such as transgenic mice, this may take some time.

**Environmental influences**

The expression of certain forms of P450 can be profoundly influenced by exposure to polycyclic aromatic hydrocarbons, barbiturates, peroxisome proliferators such as clofibrate, and a wide range of other chemicals. In most cases these effects are at the transcriptional level and appear to be related to the role of the P450s as an adaptive response system to chemical exposure. Recent work has suggested that there may be some unifying features in this induction process. The mouse peroxisome proliferator activated receptor (mPPAR), which is implicated in clofibrate acid induction, has been cloned and is a member of the nuclear steroid hormone receptor superfamily; the aromatic hydrocarbon (Ah or dioxin) receptor is also believed to be in this case also also requires an accessory protein for activity (20, 21).

The finding of a conserved 17 bp DNA sequence upstream of the phenobarbital (PB)-inducible rat liver CYP2B1 and CYP2B2 genes which is also present in the barbiturate-inducible genes from *Bacillus megaterium* (i.e. P450_{Bm1} and P450_{Bm3}) is especially intriguing (22). This conserved sequence can bind to proteins present only in PB-induced rat liver nuclear extracts and not in uninduced extracts. In contrast, the sequence binds to a protein present in uninduced, but not in barbiturate-induced *B. megaterium*; however, the effect in both systems is to increase transcription. Such a highly conserved effect from such widely divergent species suggests that there may be some underlying physiological significance of this induction; possibly there are as yet unknown endogenous inducing molecules whose effect is mimicked by barbiturates in both the mammalian and bacterial systems. Once the factors controlling the expression of individual P450 genes is better understood, the combination of the effects of environmental chemicals and an individual's genotype on susceptibility to chemical carcinogenesis will begin to be understood. It is important to note that P450 expression is regulated not purely by the rate of transcription; there are now several examples where P450 content and activity are regulated at the translational or post-translational level.

**What is the true role of cytochrome P450?**

There has been much speculation on the evolution of mammalian P450s. One postulate is that the large number of closely related forms has arisen from selective pressure during 'plant–animal warfare' so that otherwise toxic plant secondary metabolites can be metabolized (2). Because adaptation to our environment plays a major role in evolution, the diversification of the P450 into a series of multigene families to detoxify the vast number of environmental chemicals to which we are exposed is clearly justified. This, however, begs the question of what was the original role and what are the other roles that P450s have in mammals. It could be that the so-called drug-metabolizing P450s have all been recruited from ancestral genes whose reaction products have more central roles in metabolism, growth and development. Their current role in the metabolism of steroid hormones may be evidence for this. There is also evidence that this is the case for the ethanol-inducible CYP2E1, which appears to play an important role in the metabolism of endogenously produced chemicals such as ethanol and acetone, as well as in the metabolism of nitrosamines to their carcinogenic form. Many important signal transduction molecules, particularly those acting through the steroid hormone receptor multigene family, are cytochrome P450 substrates, though the physiological role of P450s in modulating signal transduction remains unclear. The years ahead will undoubtedly reveal further instances of P450s, normally considered as being 'drug-metabolizing', being involved in the biosynthesis and breakdown of many small molecules which serve as signals for growth, differentiation, morphogenesis, cell signalling and so on (23). Such functions may also be of central importance in tumour promotion and progression, indicating another interface in the carcinogenic process where the P450 system may be important.

**Conclusions**

The use of molecular biological approaches in studying P450s is beginning to shed light on the role of individual enzymes in chemical carcinogenesis. Combinations of molecular-genetic and genetic approaches will help unravel the relative contributions of environment and P450 genotype in individual susceptibility to cancer. Based on the multigenic nature of the P450 system, it is likely that many variants will be identified within the human population and undoubtedly there will be significant differences in P450 genes between ethnic groups. This has important pharmacological as well as toxicological implications. Much has still to be learnt of the role of P450s in carcinogen activation and tumour progression, and in other areas of metabolism, growth and differentiation. As a final note, the determination of the tertiary structure of a P450 involved in the activation of xenobiotics is long awaited. With this information a truly molecular understanding of the production of carcinogens by P450s will be achieved.

**References**


