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Accelerated Publications

Characterization of Ethanol-Inducible Human Liver *N*-Nitrosodimethylamine Demethylase†

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ABSTRACT: Through the use of monospecific antibodies directed against hepatic cytochrome P-450j, an enzyme induced in rats treated with ethanol or isoniazid, we have purified from human liver the related cytochrome P-450 termed HLj. HLj resembles rat P-450j and P-450 LM3a, the homologous cytochrome in rabbit liver, in its NH_2-terminal amino acid sequence, in being in highest concentration in liver microsome samples prepared from two patients intoxicated by ethanol and one patient given isoniazid, and in catalyzing the metabolic activation of the procarcinogen *N*-nitrosodimethylamine. Furthermore, each of nine human liver RNA samples contained a species of mRNA hybridizable to a cloned HLj cDNA. We conclude that HLj is related by structure, function, and some regulatory characteristics to rat P-450j and rabbit P-450 LM3a, cytochromes critical for metabolism of several clinically relevant cytotoxic and carcinogenic agents.

Many carcinogens and cytotoxic agents produce toxic effects through a process requiring oxidation of the agent by cytochromes P-450. Multiple forms of cytochrome P-450 have been identified prominently in the endoplasmic reticulum of the liver and in lesser amounts in most other tissues (Gram, 1980; Black & Coon, 1986). These isozymes differ in primary structure, in substrate specificity, and in response to inducing agents (Black & Coon, 1986; Ryan et al., 1982). An important advance in this area was the recent purification of homologous liver microsomal hemoproteins, cytochrome P-450 LM3a from the rabbit (Koop et al., 1982) and P-450j from the rat (Ryan et al., 1985). These cytochromes efficiently catalyze the metabolic activation of the procarcinogen *N*-nitrosodimethylamine (NDMA)[1] (Yang et al., 1985a,b; Ryan et al., 1986; Levin et al., 1986) and the cytotoxin carbon tetrachloride (Johansson & Ingelman-Sundberg, 1985; English & Anders, 1985). Cytochromes P-450j and LM3a are induced by commonly encountered substances such as ethanol (Koop et al., 1982, 1985; Ryan et al., 1986) and the antituberculosis agent isoniazid (INH) (Ryan et al., 1985; Koop et al., 1985). An unanswered question at present is to what extent the rapidly accumulating information about P-450j and LM3a is applicable to man and, hence, to human toxicology. In this study, we have taken advantage of the recent availability of monospecific antibodies to P-450j to establish the existence, distribution, and characteristics of the related human liver cytochrome P-450.

Experimental Procedures

Human Liver Specimens. Specimens from 20 patients were obtained at surgery under protocols approved by the Committee for the Conduct of Human Research at the Medical

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[1] Abbreviations: NDMA, *N*-nitrosodimethylamine; INH, isoniazid; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; BP, benzo[*a*]pyrene; IgG, immunoglobulin G; kDa, kilodalton(s); kb, kilobase(s).

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Table I: Patient History and Immunoquantitation of Hepatic HLj, HLp, and HLd[a]

patient no.	HLj	HLd	HLp	gender	age	smoking habits[b]	source[c]	drugs[d]
2	265	42	275	M	69	nonsmoker	L	erythromycin base, neomycin
3	453	71	150	M	27	unknown	D	ethanol (0.22%), vitamin K
4	188	66	650	F	33	unknown	D	dexamethasone, phenobarbital, diphenylhydantoin
6	165	84	200	M	26	unknown	D	hydrocortisone, cimetidine
7	100	55	175	F	48	nonsmoker	L	none
8	318	45	563	M	52	light smoker, 3 pk yr	D	dexamethasone, phenobarbital, diphenylhydantoin
9	352	85	313	M	36	unknown	D	dexamethasone, diphenylhydantoin, amobarbital
10	235	98	225	F	36	nonsmoker	L	none
11	353	77	525	F	38	unknown	D	dexamethasone, diphenylhydantoin, furosemide, sulfamethoxazole, trimethoprim, gentamycin
12	158	33	100	M	46	25 pk yr	L	none
13	BD[e]	11	763	M	47	37 pk yr	L	triacetyloleandomycin
14	100	100	100	M	50	nonsmoker	L	flurazepam
16	159	42	179	F	45	unknown	D	none
17	565	6	116	M	56	unknown	D	ethanol (0.30%), allopurinol
18	247	105	233	M	26	unknown	D	none
19	118	152	106	M	69	nonsmoker	L	none
20	160	23	309	F	51	nonsmoker	L	erythromycin base
21	182	15	163	F	41	unknown	D	hydrochlorothiazide
22	588	14	167	F	43	nonsmoker	L	isoniazid, dexamethasone (10 mg, 36-h preop)
23	194	64	113	M	47	unknown	L	none

[a] Quantitations of the human liver cytochromes P-450 were performed as described under Experimental Procedures. Densitometric values for specimen 14 were arbitrarily designated as 100%. [b] pk yr is defined as the equivalent of a package of cigarettes per day for 1 year. [c] Sources of human liver specimens were patients undergoing hepatic lobectomy (L) or brain dead renal transplant donors (D). [d] Medications received only on the day of surgery are not included. [e] BD, below limit of detection.

College of Virginia. All patients received atropine prior to general anesthesia and had normal serum transaminase and bilirubin levels. Patient code numbers refer to individual liver specimens with some specimens having appeared in other studies (Wrighton et al., 1986; Watkins et al., 1985; Molowa et al., 1986). Drugs received by the patients are indicated in Table I. Microsomes were prepared and stored as previously described (Watkins et al., 1985). Protein concentration was determined colorimetrically (Lowry et al., 1951).

Antibody Preparation. Rabbit polyclonal antibodies against purified rat P-450j (Ryan et al., 1985) were prepared as previously described (Ryan et al., 1986). The antibody was rendered monospecific for rat P-450j by absorption against solubilized microsomes from Aroclor-1254 treated rats bound to Sepharose 4B (Pharmacia, Piscataway, NJ). This monospecific anti-P-450j preparation was shown not to react with rat cytochromes P-450a–i and P-450k by enzyme-linked immunoadsorbent assay (data not shown). Murine monoclonal anti-HLp (Wrighton et al., 1986) and rabbit polyclonal anti-P-450d(–c) (Reik et al., 1982) were prepared as previously described.

Immunoblot Analysis. Immunoblot analyses were performed as described in detail elsewhere (Watkins et al., 1985). Briefly, microsomal proteins were electrophoretically separated in 1.5 mm × 10 cm sodium dodecyl sulfate (SDS)–polyacrylamide (10%) slab gels and then quantitatively transferred to nitrocellulose sheets (Bio-Rad, Richmond, CA). The sheets were blocked overnight at room temperature in phosphate-buffered saline containing 3% bovine serum albumin and 10% calf serum and then reacted with rabbit primary antibodies, followed sequentially by goat anti-rabbit IgG (Miles-Yeda, Rehovot, Israel) and peroxidase–rabbit anti-peroxidase IgG (Miles-Yeda), and finally with 3,3′-diaminobenzidine tetrahydrochloride in hydrogen peroxide (0.006%). When the primary antibodies were murine monoclonals, the second antibody was peroxidase-conjugated rabbit anti-mouse IgG (Miles-Yeda). The density and total area of the immunostained bands were determined with a Zeiss scanning densitometer (Wrighton et al., 1986). In preliminary analyses, dilution of a given microsomal sample produced densitometric values directly proportional to the amount of applied protein for all of the antibodies used.

Immunoaffinity Purification. Anti-P-450j (200 mg) was bound to CNBr-activated Sepharose CL-4B (38 mL) (Pharmacia), the remaining active groups were blocked with ethanolamine, and the matrix was washed as previously described (Thomas et al., 1979; Wrighton et al., 1986). The column was equilibrated in 50 mM potassium phosphate buffer (pH 7.4) containing 100 mM KCl, 20% glycerol, 0.2% Lubrol PX, 0.5% sodium cholate, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 mM dithiothreitol, and 1.1 g of microsomal protein from specimen 17 was solubilized in equilibration buffer containing 0.1 mM phenylmethanesulfonyl fluoride. The solubilized material (15 mg/mL) was applied to the column (0.5 mL/min), the column was washed sequentially with various buffers, and the specifically bound protein was eluted as described (Wrighton et al., 1986). The fractions containing absorbance at 280 nm were pooled, dialyzed against 40 mM sodium phosphate (pH 7.0) containing 20% glycerol, 0.01 mM EDTA, and 0.1 mM dithiothreitol, and concentrated by ultrafiltration (Amicon PM-10 membrane). The purified protein was precipitated with acetone (Haniu et al., 1984) and redissolved in 200 μL of hexafluoroacetone trihydrate, and NH_2-terminal sequence was determined by automated Edman degradation with a gas-phase sequencer as previously described (Hawke et al., 1985).

Enzyme Assays. The rates of metabolism of [^{14}C]NDMA (1 mM) and BP (80 μM) by 0.3 mg of human liver microsomal protein (10 min) were determined as previously described (Levin et al., 1986; Ryan et al., 1986).

Isolation of cDNA. Approximately 10^5 recombinants from a λgt11 library of cDNAs (Meloy Laboratories) constructed from the mRNA from a single human liver were screened with monospecific anti-rat P-450j IgG. Positive colonies were purified by multiple rescreening of isolated plaques, and phage DNA from plaque HL-P-450j was isolated by the plate lysate method (Maniatis et al., 1982). To verify the identity of HL-P-450j, the cloned cDNA insert was excised by *Eco*RI digestion and further digested with *Bam*H1 and *Stu*1, restriction sites unique to the 5′ and 3′ portions, respectively,

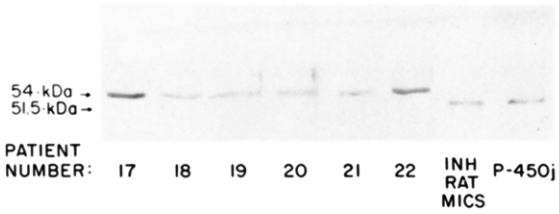

FIGURE 1: Immunoblot of liver microsomes isolated from the indicated patients and from a rat treated with INH and of purified rat P-450j developed with anti-P-450j. Liver microsomes isolated from the indicated human specimens (35 μg of protein) and an INH-treated rat (4 μg) and of purified rat P-450j (0.2 μg) were immunoblotted as described under Experimental Procedures with rabbit anti-P-450j.

of the HLj cDNA sequence. The digested DNA was subjected to Southern blot analysis (Maniatis et al., 1982) and hybridized with phP450j, a full-length HLj cDNA (Song et al., 1986). Positive signals for all restriction fragments revealed that HL-P-450j represents the middle portion of the HLj mRNA sequence (data not shown).

Northern Blot Analysis. Total RNA was extracted by the guanidine isothiocyanate method (Molowa et al., 1986) from rat and human liver. The RNA was subjected to electrophoresis in 1.0% agarose gels in 10 mM sodium phosphate buffer (pH 7.4) containing 1.1 M formaldehyde and transferred to nitrocellulose sheets (Molowa et al., 1986). As previously described (Molowa et al., 1986), the sheets were prehybridized, hybridized with [^{32}P]cDNA insert excised from clone HL-P-450j, and radioactively labeled by nick translation, and the bands were visualized by autoradiography.

Results and Discussion

We subjected samples of microsomes prepared from human liver specimens to electrophoresis in SDS–polyacrylamide gels and then transferred the separated proteins electrophoretically to nitrocellulose paper. When the immobilized proteins were exposed to anti-P-450j IgG, one well-defined band having the mobility of a 54-kDa protein was detected (Figure 1). Analysis on the same immunoblot (Figure 1) of liver microsomes prepared from an INH-treated rat and of purified P-450j revealed a single band migrating at 51.5 kDa. On similar immunoblots, the human liver 54-kDa protein immunochemically related to rat P-450j (hereafter termed HLj) was detectable in each of 20 specimens, except patient 13 (data not shown). We used scanning densitometry to quantitate the relative amounts of HLj in the specimens (Table I) and obtained values for patients 3, 17, and 22 significantly elevated as compared with those for the remainder of this population [exceeded tolerance limits: 206 ± 237, $n = 16$, $P = 0.95$, $\gamma = 0.95$ (Dixon & Massey, 1957)]. Medical records (Table I) indicate that patients 3 and 17 had intoxicating concentrations of ethanol in their blood (0.22 and 0.30 g/dL, respectively) measured shortly before surgery and patient 22 had received 600 mg of INH over the 48 h prior to surgery. Thus HLj may be inducible in man by ethanol and by INH, two of the known inducers of P-450j in the rat and of LM3a in the rabbit (Koop et al., 1982, 1985; Ryan et al., 1985, 1986). Further evidence for specific effects of these inducers on HLj is the lack of correlation in the human liver samples between the concentrations of HLj protein and those of HLd (Wrighton et al., 1986) or HLp (Watkins et al., 1985), two other well-defined human liver cytochromes P-450 (Table I). Furthermore, HLj levels do not appear to be related to the age, gender, or smoking habits of the patients (Table I).

To supplement the immunochemical evidence for homology between HLj and P-450j (Figure 1), we purified HLj by immunoaffinity chromatography. Immunoaffinity-purified HLj migrated in SDS–polyacrylamide gels as a single 54-kDa protein (data not shown) and strongly reacted with anti-P-450j IgG in immunoblot analyses (data not shown). NH_2-terminal amino acid sequence analyses demonstrated that HLj is 67% and 61% homologous with rat P-450j and rabbit LM3a, respectively (Figure 2), through their first 18 amino acids but is less than 28% homologous to 27 other cytochrome P-450 proteins (Black & Coon, 1986).

Cytochrome	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
HLj	Ala	Ala	Leu	Gly	Val	Thr	Val	Ala	Leu	Leu	Val	Trp	Ala	Ala	Phe	Leu	Leu	Leu
P-450j	Ala	Val	Leu	Gly	Ile	Thr	Ile	Ala	Leu	Leu	Val	Trp	Val	Ala	Thr	Leu	Leu	Val
LM3a	Ala	Val	Leu	Gly	Ile	Thr	Val	Ala	Leu	Leu	Gly	Trp	Met	Val	Leu	Leu	Leu	Phe

FIGURE 2: NH_2-terminal amino acid sequences of P-450 HLj, rat P-450j, and rabbit LM3a. The NH_2-terminal sequence of immunoaffinity-purified HLj was determined as previously described (Haniu et al., 1984; Hawke et al., 1985) with a 60% yield for the NH_2-terminal alanine.

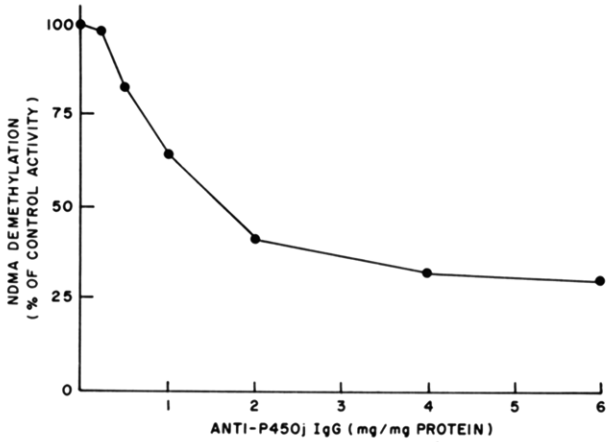

FIGURE 3: Effect of anti-P-450j IgG on the demethylation of NDMA by liver microsomes isolated from patient 17. The metabolism of [^{14}C]NDMA (1 mM) to [^{14}C]formaldehyde was determined as described under Experimental Procedures for 0.3 mg of liver microsomal protein in the presence of the indicated amounts of anti-P-450j IgG. Data are expressed as a percent of the activity obtained with incubations containing preimmune IgG [2.0 nmol of formaldehyde min^{-1} (mg of protein)$^{-1}$].

Cytochromes P-450j and LM3a account for most of the metabolism of the procarcinogen NDMA by rat and rabbit liver microsomes, respectively, when tested at concentrations of NDMA relevant to those found in vivo (Yang et al., 1985a,b; Levin et al., 1986). To determine whether HLj is functionally as well as structurally related to P-450j and LM3a, we measured the rates of NDMA metabolism by several human liver microsome samples. As shown in Table II, the rates of NDMA metabolism in samples from patients who received ethanol (patient 17) or INH (patient 22) and had high levels of HLj protein (Table I) were significantly elevated [exceeded tolerance limits: 0.39 ± 0.76, $n = 4$, $P = 0.95$, $\gamma = 0.95$ (Dixon & Massey, 1957)] as compared with the rates found for patients (18–21) who had not been exposed to known inducers of P-450j and had low amounts of HLj protein (Table I). An excellent correlation ($r = 0.87$) was observed between the rates of NDMA metabolism and HLj protein levels in these samples. That NDMA demethylation was lower in patient 22 as compared with patient 17, despite similar, elevated amounts of HLj protein in these two microsomal samples (Table I), may reflect different amounts of inactive apocytochrome HLj. Most important was the demonstration that NDMA demethylase in human liver microsomes was progressively inhibited by increasing amounts of anti-P-450j antibody (Figure 3). These results indicate that HLj is responsible for at least 70% (Figure 3) of the de-

Table II: Effect of Anti-P-450j IgG on the Metabolism of NDMA or BP by Human Liver Microsomes[a]

	NDMA metabolism [nmol of HCHO formed·min^{-1}·(mg of protein)$^{-1}$]		BP metabolism [mol of 3-OH-BP formed·min^{-1}· (mg of protein)$^{-1}$]	
patient no.	control	+anti-P-450j	control	+anti-P-450j
17	2.18	0.49 (78)	57.7	57.7 (0)
18	0.56	0.16 (71)	86.8	78.6 (9)
19	0.39	0.11 (72)	78.6	75.9 (3)
20	0.30	0.09 (70)	73.2	66.8 (9)
21	0.31	0.10 (68)	75.0	65.9 (12)
22	1.13	0.26 (77)	38.0	34.0 (11)

[a] Microsomes (0.3 mg of protein) from the indicated patient were incubated with NADPH and either 1 mM NDMA or 80 μM BP as described under Experimental Procedures in the presence of 6 mg of preimmune IgG (control) or 6 mg of anti-P-450j IgG per milligram of microsomal protein. Percent inhibition by anti-P-450j IgG of the indicated metabolic activity is given in parentheses.

methylation of NDMA in microsomes from a patient intoxicated by ethanol who had high levels of immunoreactive HLj (patient 17). In additional experiments, 68–78% of the NDMA demethylase in each of six human specimens examined was inhibited by anti-P-450j IgG (Table II), even though the range of NDMA activity among these patients was 7-fold. It is possible that anti-rat P-450j is incapable of completely inhibiting the catalytic activity of the human enzyme. Thus, the contribution of HLj to NDMA metabolism in human liver could be even greater than the values reported (Table II). However, we cannot exclude the possibility that 20–30% of the NDMA metabolism in our samples reflects other cytochromes P-450 whose abundance coincidently parallels that of HLj. We can rule out nonspecific inhibition of cytochromes P-450 by anti-P-450j IgG as a likely explanation for these results because the rates of oxidation of another procarcinogen, benzo[*a*]pyrene (BP), were unrelated to the amounts of HLj protein in the microsomal samples and were unaffected by additions of anti-P-450j IgG to the reaction mixture (Table II). The results of our experiments with human liver are consistent with evidence from animal studies indicating that oxidation of BP is supported by isozymes of cytochrome P-450 other than P-450j (Ryan et al., 1982, 1985). Therefore, we conclude that HLj is the isozyme of human liver cytochrome P-450 primarily responsible for the metabolism of NDMA to its carcinogenic metabolite.

In a final series of experiments, we used immunochemical screening to select, from a human liver cDNA library cloned in the expression vector λgt11, a recombinant phage harboring a 1.0-kb cloned cDNA that directed the synthesis of a fusion protein recognized by anti-P-450j IgG. Moreover, the identity of this cloned cDNA (HL-P-450j) as HLj was confirmed by demonstrating that fragments produced by digestion of HL-P-450j with selected restriction endonucleases hybridized with a full-length HLj cDNA of known sequence (Song et al., 1986). Analyses of human liver RNA on Northern blots revealed that our HLj cDNA hybridized to a mRNA species having an apparent size of 1.8 kb in each of the nine samples examined (Figure 4). A similar hybridization signal was found in RNA from the liver of an untreated rat (Figure 4). We estimated the amounts of HLj mRNA by quantitative scanning densitometry of this Northern blot and normalized each result to the amount of RNA hybridizing to a probe for a common liver gene, B-actin (not shown). The amount of HLj mRNA showed no correspondence to the amount of HLj protein in the same human liver (Figure 4, Table I). This is not the result of technical artifacts because in other studies

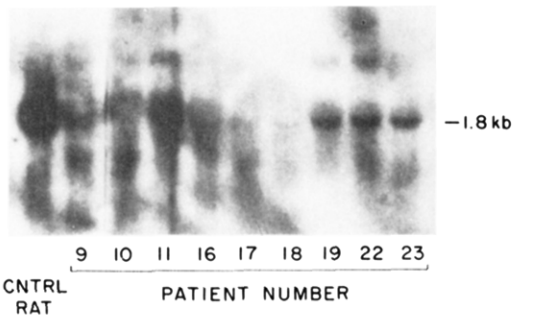

FIGURE 4: Northern blot hybridization of human and rat liver RNA with HLj cDNA. Samples of RNA extracted from the indicated human liver specimens (50 μg) and the liver of control rat (50 μg) were separated by electrophoresis, transferred to nitrocellulose, and hybridized with [^{32}P]cDNA insert excised from clone HL-P-450j as described under Experimental Procedures.

of the same RNA samples analyzed in a similar manner for the amounts of cytochrome P-450 HLp, we found that HLp mRNA is directly proportional to the amount of HLp protein (Molowa et al., 1986). Furthermore, when rats were treated with an inducer of P-450j, there was an increase in P-450j protein without a corresponding increase in the amounts of P-450j mRNA (Song et al., 1986). Therefore, unlike the regulation of HLp, control of HLj protein appears to involve processes unrelated to accumulation of HLj mRNA transcripts.

The presence in human liver of a cytochrome P-450 (HLj) similar to P-450j and LM3a in structure, catalytic activity, and regulatory characteristics could explain a number of hepatotoxic reactions in man. For example, HLj appears to be inducible by ethanol (Table I) and may, like LM3a, rapidly metabolize acetaminophen (Morgan et al., 1983). The hepatotoxicity of therapeutic levels of acetaminophen in alcoholics (Seeff et al., 1986) may be due to enhanced conversion of acetaminophen to toxic metabolites catalyzed by elevated levels of HLj. Likewise, because LM3a and P-450j metabolize carbon tetrachloride (Johansson & Ingelman-Sundberg, 1985; English & Anders, 1985) and are induced by treatment of rabbits or rats with 2-propanol or ethanol (Ryan et al., 1986; Koop et al., 1985), the reported human hepatoxicity from exposure to carbon tetrachloride in workers previously exposed to 2-propanol or ethanol (Folland et al., 1976) may reflect induction of HLj and, thus, enhanced activation of carbon tetrachloride to cytotoxic metabolites. Finally, since INH, an inducer of P-450j in rats, also induces the metabolism of acetylhydrazine (a metabolite of INH) to a hepatotoxic intermediate, the subclinical hepatocellular damage observed in many patients treated with INH may involve drug-mediated induction of HLj (Nelson et al., 1976).

Also of potential clinical significance is our finding that HLj, like LM3a and P-450j, is largely responsible for the bioactivation by human liver microsomes of NDMA to its ultimate carcinogen. Nitrosamines are widely distributed in the environment, having been detected in food, drink, tobacco, and air, in addition to being formed endogenously in the gut from amines and nitrites (Preussmann, 1984). Ethanol consumption increases the incidence of human cancer possibly as a result of altering the pharmacokinetics of nitrosamines (Swann, 1984). Thus, the regulation of HLj in hepatic and possibly in extrahepatic tissues by ethanol and other inducers may play a critical role in nitrosamine carcinogenesis. The ability to quantitate HLj and its mRNA offers an opportunity to investigate interindividual differences among people for an enzyme involved in potential adverse reactions to common toxic and carcinogenic substances.

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