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Heme Enzyme Patterns in Rat Liver Nodules and Tumors[1]

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ABSTRACT

Chemically induced rat hepatocyte nodules and carcinomas have a reduced capacity to oxidize drugs. The reduction in monooxygenase activity results largely from the partial loss of cytochrome P-450, a heme-containing terminal electron acceptor. To determine whether the cytochrome P-450 deficit was indicative of an altered heme metabolism, we quantitated four heme-containing proteins in normal rat liver and in rat liver nodules and cancers induced by 2-acetylaminofluorene or diethylnitrosamine: cytochrome P-450; cytochrome b_5; catalase (EC 1.11.1.6); and tryptophan 2,3-dioxygenase (EC 1.13.11.11). The amounts of these components in nodules were 45%, 88%, 50%, and 59% of normal liver, respectively; in 2-acetylaminofluorene-induced cancers, 65%, 74%, 64%, and 65%, respectively; and in diethylnitrosamine-induced cancers, 40%, 69%, 56%, and 52%. δ-Aminolevulinic acid synthase (EC 2.3.1.37), the rate-limiting enzyme in the heme synthetic pathway, and heme oxygenase (EC 1.14.99.3), a degradative enzyme, were also quantitated. The amounts of these enzymes in nodules were 95% and 138% of normal liver, respectively, whereas in 2-acetylaminofluorene-induced cancers, they were 47% and 233%, and in diethylnitrosamine-induced cancers, they were 50% and 175%. These data indicate that four nonmitochondrial liver hemoproteins were diminished to about the same extent in hepatic nodules and cancers. Nodules and cancers also demonstrated an increased capacity for heme degradation, while cancers also demonstrated a decreased capacity for heme synthesis. Thus, the resistance of nodules and tumors to P-450-activated cytotoxic agents may ultimately result from a disturbance in heme metabolism.

INTRODUCTION

A consistent feature of the chemically induced preneoplastic hepatocytes is their relative resistance to hepatotoxins (1, 2). This characteristic has been suggested to offer a selective advantage in a toxic environment such that these focal cells may proliferate rapidly in response to chemically induced hepatocellular injury and ultimately give rise to cancer (3, 4). The basis for their resistance to hepatotoxins resides in metabolic alterations that diminish the cell's capacity to activate xenobiotics to reactive species (5) and is largely the result of reduced amounts of P-450,[3] the terminal electron acceptor of the monooxygenase system (6–8). However, these cells also demonstrate enhancement of various other detoxication mechanisms (9, 10).

Because diminution of the heme protein P-450 has been a constant feature of preneoplastic HN and PHC, we undertook to determine whether altered heme metabolism could be the basis of the P-450 deficit. In a previously reported study of mouse liver cancers (11), we examined four heme-containing proteins representing three cellular compartments: microsomal cytochromes P-450 and b_5; cytosolic TRYPD; and CAT, a peroxisomal enzyme. We also measured the enzymes ALAS, the rate-limiting heme synthetic enzyme, and MHO, a microsomal enzyme that degrades heme. We found that all of the hemoproteins examined were diminished in the cancers. These tissues also demonstrated reduced levels of ALAS, whereas the levels of MHO were increased. Taken together, these findings suggested that the decline in hemoprotein levels during hepatocarcinogenesis may result from a diminution of the intracellular heme pool, due either to reduced heme synthesis or increased heme catabolism or both.

Because mouse liver cancers demonstrate some enzyme alterations that are not consistent with those seen in rat HN and PHC (12), we extended our study to rat liver lesions to determine whether such alterations are found in that model as well. (In rat HN and PHC, the specific activities of epoxide hydrase and glucuronsyltransferase levels are significantly greater than in normal liver. Mouse liver lesions, in contrast, demonstrate significantly reduced glucuronsyltransferase and moderately reduced epoxide hydrase.) In the study that we report here, the same components that we examined in mouse liver cancers were examined in HN induced with AAF and in PHC induced with AAF or DEN. RL was also included in this study to determine whether HN and PHC enzyme patterns are a reflection of the proliferative state. We now report that rat HN and PHC, like the mouse lesions, demonstrated a marked diminution of the four nonmitochondrial hemoproteins and increased MHO. In addition, PHC demonstrated a reduced capacity for heme synthesis.

MATERIALS AND METHODS

Chemicals. Ascorbic acid, ATP, bilirubin, bovine serum albumin, horse methemoglobin, Coenzyme A, Ehrlich's reagent, glycine, hemin, human albumin, NADH, NADPH, pyridoxal phosphate, succinic acid, and tryptophan were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals. Male CD Sprague-Dawley rats were obtained from Charles River Breeding Laboratory (Wilmington, MA) at approximately 100 g of body weight. Rats treated with DEN (80 ppm in drinking water for 12 wk) and the untreated control group were maintained on Purina laboratory chow. AAF was administered to rats by cyclic feeding of Altromin C 1000 purified diet containing 0.06% AAF, Altromin Tier-Labor Service (Laga, Federal Republic of Germany). Following AAF treatment, the animals were maintained on AAF-free C 1000 diet as were untreated control animals. Partial hepatectomy was performed under general anesthesia on age-matched rats (untreated controls) 22 and 40 h prior to sacrifice. HN and PHC from both treatment groups were harvested 2 to 4 mo after the last exposure to carcinogen.

Tissue Preparation. HN and PHC 1 cm or greater in diameter were used from several rats to form pools weighing 8 g or more. The tissues were homogenized in 4 volumes of cold 0.25 M sucrose-0.1 mM pyridoxal phosphate-20 mM Tris-HCl, pH 7.4. An aliquot of the homogenate was diluted with 4 volumes of 50 mM phosphate buffer, pH 6.8, and after deoxycholate was added to 0.5%, allowed to stand at room temperature for 30 min. This fraction was subsequently centrifuged at 105,000 × *g* for 60 min, and the supernatant portion was assayed for CAT activity. The remaining homogenate was centrifuged at 10,000 × *g* for 10 min. The resulting pellet was homogenized in 2 volumes of homogenizing medium and assayed for ALAS. The 10,000 × *g* supernatant fraction was centrifuged at 105,000 × *g* for 60 min to sediment the microsomes which were then washed by resuspension in 0.15 M KCl and centrifuged again at 105,000 × *g* for 30 min. The microsomal suspension was assayed for heme oxygenase activity, P-450, and b_5

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[3] The abbreviations used are: P-450, cytochrome P-450; AAF, 2-acetylaminofluorene; ALAS, δ-aminolevulinic acid synthase; b_5, cytochrome b_5; CAT, catalase; DEN, diethylnitrosamine; HN, hepatic nodule; MHO, microsomal heme oxygenase; PHC, primary hepatocellular carcinoma; RL, regenerating liver; TRYPD, tryptophan dioxygenase.

content. The postmicrosomal fraction was assayed for TRYPD.

Enzyme Assays. ALAS was determined according to the procedure described by Burnham (13). An extinction coefficient of 58 $mM^{-1}cm^{-1}$ was used to calculate ALAS concentration. CAT was measured according to Bergmeyer *et al.* (14) with samples prepared as described by Reddy *et al.* (5). An extinction coefficient of 0.040 $\mu M^{-1}cm^{-1}$ was used to calculate H_2O_2 concentration. Cytochrome b_5 was determined according to Oyanagui *et al.* (8). P-450 was determined by the dithionite-CO absorbance difference, as described by Omura and Sato (16). MHO was assayed by the method of Tenhunen *et al.* (17). A crude preparation of rat liver biliverdin reductase (the 40 to 60% ammonium sulfate fraction) was prepared according to the method of Tenhunen *et al.* (18) and substituted for cytosol as a source of biliverdin reductase. Bilirubin concentrations were determined from standard curves generated by adding known amounts of bilirubin to cold incubation mixtures of identical composition. TRYPD was assayed according to the method of Knox *et al.* (19). Cytosol was preincubated with heme to saturate the apoenzyme with heme prior to assay. Change in absorbance was measured at 360 nm, and an extinction coefficient of 4.53 $mM^{-1}cm^{-1}$ was used to calculate kinurenine concentration. The protein concentration of the resuspended 10,000 × *g* pellet was determined by the method of Lowry *et al.* (20). The method of Bradford (21) was used to determine protein in the microsomal and postmicrosomal fractions. Bovine serum albumin was used as a standard in both methods of determination.

RESULTS

Table 1 contains the hemoprotein data from the three experimental groups that were treated with AAF to produce HN and PHC or DEN to produce PHC. Each of the hemoproteins was diminished in both lesions, but no pattern was evident that distinguished HN from PHC. P-450, which was present in amounts that ranged from 40 to 65% of control values, was the most affected hemoprotein, and b_5, present in amounts that were 69 to 88% of control, was least affected. The specific activities of CAT and TRYPD ranged from 50 to 64% and 52 to 65%, respectively, in these lesions.

The data on the two heme-metabolizing enzymes are also shown in Table 1. Unlike the hemoproteins, these two enzymes demonstrated greater alteration in PHC than in HN. ALAS activity in HN was 95% of control, but in PHC, it was decreased to 50%. MHO was 138% of control in HN, and in PHC, 175 to 233%.

RL was also included in this study to determine whether HN and PHC enzyme patterns were a reflection of the proliferative state. Data obtained at 22 and 40 h following hepatectomy are shown in Table 2. At both time points, P-450, b_5, and CAT were diminished to levels near those seen in HN and PHC. In contrast to HN and PHC, however, RL demonstrated elevated TRYPD. As seen in HN, ALAS was somewhat diminished at 22 h, but in contrast, it was increased at 40 h. MHO was increased in RL to levels that were, on the average, greater than those in PHC.

DISCUSSION

Decreased levels of P-450 and a reduced capacity to oxidize xenobiotics are characteristic of HN and PHC (6–8). Seventy % of the heme synthesized by the hepatocyte is utilized for P-450 (22, 23). Thus, reduced levels of P-450 suggested that HN and PHC may not synthesize sufficient heme to maintain P-450 at normal levels. This idea is supported by our recent finding that four hemoproteins in three different cellular compartments in mouse liver cancers were diminshed (11). The additional findings of reduced ALAS and increased MHO in these lesions further support the idea that hemoprotein decline during hepatocarcinogenesis may result from a reduction in the intracellular heme pool, either because of reduced heme synthesis or increased heme catabolism or both. That altered heme metabolism and hemoprotein decline occur in both spontaneous and chemically initiated mouse liver cancers suggests that altered heme metabolism is linked to the earliest stage of carcinogenesis, that stage termed initiation. Because the enzyme patterns seen in the mouse liver cancers differed from those seen in rat HN and PHC (12), we extended the study to rat to determine whether broad hemoprotein decline and altered heme metabolism during hepatocarcinogenesis were unique to the mouse or whether rat HN and PHC demonstrated a similar pattern. Similar findings in the rat lesions would support our concept that altered heme metabolism may be a part of the genetic program for carcinogenesis linked to initiation.

We demonstrated that, in addition to P-450, three other hemoproteins were also reduced in AAF- and DEN-induced rat hepatic lesions. The basis for the partial loss of these proteins is as yet unknown. As we suggested previously, diminished ALAS and elevated MHO in PHC could result in limiting amounts of available heme, due either to reduced heme synthesis or to increased heme catabolism with which P-450 decline has often been reported (24, 25). Although HN and PHC demonstrated comparable diminution of the four hemoproteins, ALAS and MHO levels were not as markedly changed in HN as in PHC, suggesting that the hemoprotein diminution in HN, and perhaps in PHC also, may be attributable to some factor other than altered heme metabolism. Because we examined only one of eight enzymes in the heme biosynthetic pathway, the possibility still remains that an enzyme defect elsewhere in this pathway restricts heme synthesis in HN and PHC.

Findings reported by other investigators suggest that dimin-

Table 1 *Heme enzymes in rat liver nodules and PHC*

	P-450 (nmol/mg)	b_5 (nmol/mg)	CAT (μmol/mg/min)	TRYPD (nmol/mg/h)	ALAS (nmol/mg/h)	MHO (nmol/mg/h)
Experiment 1 (AAF treatment)						
Nodule (3)[a]	0.590 ± 0.254[b, c]	0.557 ± 0.116	0.430 ± 0.350[d]	89.22 ± 18.80[c]	0.578 ± 0.185	9.86 ± 4.10
Liver (3)	1.297 ± 0.126	0.630 ± 0.078	0.867 ± 0.046	151.65 ± 16.63	0.607 ± 0.153	7.13 ± 0.86
Experiment 2 (AAF treatment)						
PHC (2)	0.731 ± 0.086[d]	0.430 ± 0.024	0.460 ± 0.123	84.97 ± 0.92[d]	0.444 ± 0.060[d]	12.32 ± 0.59[c]
Liver (2)	1.132 ± 0.109	0.581 ± 0.112	0.722 ± 0.115	131.51 ± 16.54	0.940 ± 0.187	5.28 ± 1.18
Experiment 3 (DEN treatment)						
PHC (2)	0.432 ± 0.074[c]	0.378 ± 0.165	0.402 ± 0.030[d]	78.72 ± 4.95[d]	0.357 ± 0.032	12.32 ± 2.52[d]
Liver (2)	1.076 ± 0.015	0.544 ± 0.034	0.720 ± 0.110	151.07 ± 23.02	0.716 ± 0.212	7.04 ± 0.40

[a] Numbers in parentheses, number of tissue pools examined.
[b] Mean ± SD.
[c] Different from control ($P < 0.01$, *t* test).
[d] Different from control ($P < 0.05$, *t* test).

Table 2 *Heme enzymes in regenerating rat liver*

	P-450 (nmol/mg)	b_5 (nmol/mg)	CAT (μmol/mg/min)	TRYPD (nmol/mg/h)	ALAS (nmol/mg/h)	MHO (nmol/mg/h)
22 h post-partial hepatectomy (2)[a]	0.692 ± 0.124[b-d]	0.316 ± 0.049	0.640 ± 0.260	254.49 ± 30.11[c]	0.715 ± 0.001	11.82 ± 1.50[c, d]
40 h post-partial hepatectomy (3)	0.978 ± 0.213	0.383 ± 0.056	0.568 ± 0.061	280.89 ± 22.06	1.001 ± 0.167	15.75 ± 0.98
Control liver (3)	1.123 ± 0.311	0.626 ± 0.175	0.800 ± 0.012	158.62 ± 10.92	0.803 ± 0.084	5.28 ± 0.98

[a] Numbers in parentheses, number of tissue pools examined.
[b] Mean ± SD.
[c] Differs from control ($P < 0.05$, t test).
[d] Differs from 40 h post-partial hepatectomy ($P < 0.05$, t test).

ished hemoprotein and altered heme metabolism may be linked to aberrant iron metabolism. HNs characteristically fail to demonstrate stainable iron when siderosis is induced in the liver by dietary iron overload (26). Also, the administration of iron chelators to rats on a low iron diet depletes ferritin iron and subsequently brings about some of the changes that are seen in HN and PHC, *i.e.*, decreased P-450 and ALAS and increased MHO (27, 28).

The diminution of hemoproteins in HN and PHC may also result from reduced synthesis of the apoproteins. This seems to be true of TRYPD. Prior to assay, the enzyme was preincubated with heme to saturate the apoenzyme. Despite this, the measurable activity of TRYPD in HN and PHC was less than control. Thus, reduced TRYPD activity cannot be attributed to the lack of heme. It more likely results from reduced amounts of the apoenzyme. The fact that some of the microsomal P-450-dependent monooxygenases are reduced more than others in HN and PHC also suggests that specific proteins are diminished. While the total amount of microsomal protein in HN and PHC is reduced (29, 30), microsomal proteins such as glucuronosyl transferase and epoxide hydrase are increased severalfold (25, 31). Obviously, this can only occur if other proteins in this membrane are reduced or lost.

Because proliferative activity within HN and PHC exceeds that of the normal liver, it is possible that the metabolic alterations seen were associated with proliferation and not directly related to the carcinogenic process. RL, HN, and PHC demonstrated substantial reductions of the three hemoproteins, P-450, b_5, and CAT, and elevation of MHO. Because these alterations were seen in all three tissues, it suggests that they may be associated with the proliferative state. This is difficult to ascertain using RL as a model for proliferation because of the magnitude of the proliferative response following partial hepatectomy and its high degree of synchrony. The picture is further confused by the fact that some of alterations seen in RL are attributable to operative stress (11, 12). However, considering the relative proliferative activity of the three tissues (RL > PHC > HN), it appears that the only alteration that corresponded closely with proliferative activity was that of MHO. Despite the fact that ALAS and MHO were altered less in HN than in PHC, hemoprotein levels were equally diminished in both lesions and in these more than in RL. Thus, despite the similarity of the phenotypic alterations seen in HN and PHC and RL, the decline in hemoproteins in HN and PHC appears to be largely attributable to some factor other than proliferation.

In conclusion, we found that the partial loss of P-450 in chemically induced HN and PHC was associated with a general decline of all hepatic hemoproteins studied. The basis for hemoprotein decline is unknown, but the altered levels of ALAS and MHO suggest that it may result from limiting supplies of heme due to reduced heme synthesis or increased heme degradation. The findings of other investigators suggest that heme synthesis may also be restricted by limited intracellular iron. In addition, from our data and those of others, it is suggested that the TRYPD and P-450 apoproteins are synthesized in reduced amounts in these lesions. HN and PHC demonstrate a broad spectrum of alterations, some of which do have an impact on xenobiotic metabolism. However, it seems unlikely that such alterations, including altered heme metabolism, represent a chemically induced response to limit xenobiotic activation through reduced monooxygenase activity. The nature of these alterations and their ubiquity suggest that they are linked to the genetic program for carcinogenesis.

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