Heme Enzyme Patterns in Genetically and Chemically Induced Mouse Liver Tumors

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ABSTRACT

Chemically induced rat hepatocyte nodules and hepatomas have repeatedly been shown to be deficient in Phase I drug-metabolizing enzymes. Some of these reduced activities are attributable to a diminution of the heme-containing terminal electron acceptor, cytochrome P-450. We recently demonstrated that spontaneous mouse liver tumors exhibit the same deficiency. Therefore, chemically induced and spontaneous liver tumors share common metabolic alterations which are likely to represent intrinsic characteristics of the tumorigenic process and are independent of its etiology. To determine whether the cytochrome P-450 deficit was the result of an altered heme metabolism, we quantitated four heme-containing proteins in normal mouse liver, spontaneous mouse liver tumors, and those induced by a single injection of diethylnitrosamine: cytochrome P-450; cytochrome b5; tryptophan 2,3-dioxygenase (EC 1.13.11.11); and catalase (EC 1.11.1.6). The amounts of these components in spontaneous tumors relative to normal liver were 0.35, 0.68, 0.76, and 0.51, respectively. Similar values were obtained with chemically induced tumors. The enzymes 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 spontaneous tumor relative to liver were 0.49 and 1.51, respectively. Again, similar values were observed for the chemically induced tumors. Alteration of the latter two enzyme activities may be sufficient for the altered hemoprotein patterns seen in mouse liver tumors. Further, this pattern of metabolic alteration is common to both chemically induced and spontaneous tumors. Thus, tumor resistance to cytotoxic agents activated by the monoxygenase system is not necessarily induced by exposure to these agents, nor as a result of selection.

INTRODUCTION

A consistent feature of the chemically induced preneoplastic liver focus is its relative resistance to hepatotoxins (1–3). This characteristic has been suggested to offer a selective advantage in a toxic environment in which these focal cells may proliferate rapidly in response to chemically induced hepatocellular injury and ultimately give rise to malignancy (4). Their resistance to hepatotoxins seems to reside in metabolic alterations that diminish the cell's capacity to activate xenobiotics to reactive species (5). This is largely attributable to reduced amounts of P-450,6 the terminal electron acceptor of the monoxygenase system (6–8). Additionally, these cells demonstrate enhancement of various other detoxifying mechanisms (9, 10).

The finding, that foci of such altered hepatocytes arise and proliferate during chronic exposure to chemical carcinogens and may regress when this exposure ceases, has given rise to the concept that these phenotypic alterations are the consequence of an adaptive response to a toxic presence. However, we have recently demonstrated a similarly altered metabolic pattern in spontaneous mouse liver tumors (11). The occurrence of such a phenotypic alteration in the absence of chemical carcinogens or promoting agents suggests that these changes are linked to the carcinogenic program itself. Because diminution of the heme protein P-450 has been a constant feature of preneoplastic liver lesions and primary hepatocellular carcinomas, we undertook a study to determine whether altered heme metabolism could be the basis of the P-450 deficit. Four heme-containing proteins representing 3 cellular compartments were examined in liver tumors of C57BL/6N × C3H/HeN F1 (hereafter called B6C3F1) mice: microsomal cytochromes P-450 and b5; cytosolic TRYPD; and CAT, a peroxisomal enzyme. In addition, we quantitated the enzymes ALAS, the rate-limiting heme synthetic enzyme, and MHO, a microsomal enzyme degrading heme. Regenerating liver and liver from sham-operated mice were also included in this study to determine whether tumor enzyme patterns are a reflection of the proliferative state. We now report that MLT, whether spontaneous or chemically induced, demonstrated a marked diminution of heme-containing enzymes and ALAS. In contrast, MHO was increased in both types of tumor.

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. Pregnant female C57BL/6N mice bearing B6C3F1 mice were obtained from the Animal Program of the Division of Cancer Treatment, National Cancer Institute, at about the 14th day of gestation and placed on a diet of Purina rodent chow (Pearland Feed Co., Pearland, TX). Hepatic tumors were induced chemically according to the regimen of Vesselinovitch et al. (12) in male pups, which were given injections of a single i.p. dose of DEN, 10 mg/kg body weight, at 15 days of age. Other pups, not treated, were maintained for the development of spontaneous tumors. Starting at 1 yr of age, each mouse was bled monthly, and its circulating level of α-fetoprotein was determined by radioimmunoassay (13, 14). Values of 1 μg/ml or higher were invariably associated with sufficient liver tumor mass for analysis. Partial hepatectomy was performed under general anesthesia 48 h before the animals' sacrifice.

Tissue Preparation. Liver and isolated MLT were homogenized in 4 volumes of cold 0.25 M sucrose containing 0.1 mM pyridoxal phosphate and 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 adding deoxycholate to 0.5% (w/v), 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 washed by resuspending in 0.15 M KCl and pelleting again by centrifugation. The microsomal suspension was assayed for MHO activity and for P-450 and cytochrome b5 content. The postmicrosomal fraction was assayed for TRYPD activity.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: P-450, cytochrome P-450; ALAS, δ-aminolevulinic acid synthase; b5, cytochrome b5; CAT, catalase; DEN, diethylnitrosamine; MHO, microsomal heme oxygenase; MLT, mouse liver tumor(s); TRYPD, tryptophan dioxygenase.

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Enzyme Assays. ALAS was determined according to the procedure described by Burnham (15), in which an extinction coefficient of 58 mm M⁻¹ cm⁻¹ is used to calculate ALAS concentration. CAT was measured according to Bergmeyer et al. (16) with samples prepared as described by Reddy et al. (17). An extinction coefficient of 0.040 μm⁻¹ cm⁻¹ was used to calculate H₂O₂ concentration. Cytochrome b₅ was described by Burnham (15), in which an extinction coefficient of 58

The protein concentration of the resuspended 10,000 x g pellet was determined by the method of Lowry et al. (22), and the method of Bradford (23) was used to determine protein in the microsomal and postmicrosomal fractions. Bovine serum albumin was used as a standard for both methods of determinations.

RESULTS

The results presented in Table 1 were obtained using 3 pools of chemically induced MLT and 3 pools of liver from age-matched controls or non-tumor-bearing lobes from MLT-bearing mice. (Although the data are not shown here, we have found and previously reported that control livers and non-tumor-bearing lobes from MLT-bearing mice have identical levels of the various enzyme activities studied.) All 4 heme-containing proteins were found to be decreased in MLT, 3 significantly. The 2 microsomal proteins P-450 and b₅ were reduced to 35% and 68%, respectively, of control values. The peroxisomal enzyme CAT was reduced to 51% of control. TRYPD, the cytosolic enzyme, was reduced to 76% of control liver values, a difference that was not statistically significant. Both enzymes involved in heme metabolism were also found to be significantly altered in MLT. ALAS, the first enzyme in the heme biosynthetic pathway, was only 49% of the control liver value, whereas MHO, the heme-decyclizing enzyme, increased to 151% of control levels.

The results presented in Table 2 were obtained from 2 pools of spontaneous MLT and 3 pools of control liver. The pattern of enzymic alteration seen in chemically induced MLT was also found in spontaneous MLT. P-450 and b₅ were reduced to 47 and 72%, respectively, of control. CAT and TRYPD values were 69 and 75% of control. ALAS and MHO were 43 and 141% of control, respectively. Except for b₅, all observed differences within this group were statistically significant.

Although the MLT cell population proliferates relatively slowly, we examined regenerating mouse liver to determine whether alterations seen in MLT might be associated with cell proliferation. Because of the large number of partial hepatectomies required to provide the necessary tissue, only one pool of regenerating liver was examined. Additionally, 2 pools each of liver from sham-operated mice and normal control mice were examined. The results are shown in Table 3. P-450 levels in both regenerating and sham-operated mouse liver were 74% of control. In contrast to MLT, b₅ was elevated to 132% of control in regenerating liver, whereas no change occurred in the sham-operated livers. CAT and TRYPD were diminished in regenerating liver to the same extent as in the induced MLT; they were, respectively, 70 and 75% of control. In the livers of sham-operated mice, CAT was unchanged, and TRYPD was 86% of control. In regenerating liver, as in MLT, ALAS was found to be reduced (to a lesser extent), whereas MHO was elevated above levels seen in MLT. The levels of these enzymes were 84 and 185%, respectively, of control. ALAS and MHO were unchanged in the livers of sham-operated mice.

DISCUSSION

Our previous finding that spontaneous MLT, like chemically induced rat hepatic tumors, was deficient in P-450 suggests that this characteristic is linked to the cellular program for carcinogenesis and is not an adaptive response to a toxic presence (11). The purpose of the present study was 2-fold: to further characterize the spontaneous MLT and to determine whether a reduced level of P-450 in the MLT might indicate an alteration in heme metabolism.

We found that the 4 heme-containing proteins examined, b₅, CAT, P-450, and TRYPD, were diminished in both spontaneous and chemically induced MLT to levels ranging from 75 to 35% of control values. MLT of both origins were found to have reduced levels of ALAS and elevated levels of MHO. This latter finding suggests that these lesions may have a reduced...
capacity for heme synthesis and an increased capacity for heme degradation, either of which may account for the diminution of heme protein. It should be reiterated that chemical induction consisted of a single dose of DEN administered 15 days after birth, many months prior to the harvesting of MLT. Although chemically initiated, the cells that ultimately gave rise to MLT progressed in the absence of any selective chemical agent, and the absence of chronic chemical exposure may account for the high degree of similarity between the 2 tumor types (24). It must also be mentioned that DEN-induced MLT appeared 5–6 mo earlier than did spontaneous MLT. Consequently, DEN-treated mice used in this study were 1–1.5 yr of age, and untreated mice were 1.5–2 yr of age. Differences between the 2 groups, seen in both MLT and liver, are likely attributable to their difference in age. Since there was no toxic environment, these findings strongly support the notion that the tumor phenotype is inherent in the genetic program for malignancy; it is not conferred by a process of adaptation to and/or selection by a chemical carcinogen. It may, however, result from either genetic or chemical “initiation” of the carcinogenic process. Furthermore, tumor growth occurred without toxic suppression of non-tumor cells.

Because tumors are made up of a dividing cell population, it is possible that the observed phenotypic alteration is a consequence of cell division and not malignancy. On first inspection, the enzyme patterns of MLT appeared similar to those of regenerating liver, both tissues demonstrating decreased amounts of P-450, TRYPD, CAT, and ALAS, and increased amounts of MHO. Sham operation, however, had a marked effect on the levels of all 4 heme-containing enzymes. Thus, some of the changes seen in regenerating liver are the result of operative stress. This factor was most significant in the case of P-450, which was found equally diminished in regenerating and sham livers. We estimated that, in the regenerating liver, the operative stress component accounted for about 50 and 25% of the total decrease in TRYPD and CAT activities, respectively. Considering the magnitude of the relative proliferative response in the regenerating liver compared with that of MLT, the enzymic alterations observed were quite small. Also, in contrast to its content in MLT, bs was elevated in regenerating liver. These data indicated that some alteration, but not all, of CAT, TRYPD, ALAS, and MHO activities in MLT may result from cell proliferation. This cannot, however, be the basis of the alterations in P-450 and bs levels in MLT. We conclude that certain phenotypic alterations observed in MLT largely reflect a pattern intrinsic to hepatic tumors, not a proliferative state.

The significance of these findings is still unknown. Decreased levels of hemoprotein and altered heme metabolism may result, in part, from partial loss of specific hepatic function during the carcinogenic process, or they may be linked to the genetic program that becomes activated with initiation by any cause, genetic or chemical. Because of the liver's tremendous capacity to metabolize xenobiotics, the normal hepatocyte contains comparatively large amounts of the various P-450 species. The normal hepatocyte's heme requirements are high. It has been estimated that 70–80% of the heme synthesized in that cell is used for microsomal P-450s (25), which have half-lives ranging from 10–20 h (26). The half-life of the heme moiety itself is even shorter than that of the apoprotein. For the species P-450b, the half-life of the heme moiety has been shown to be about one-half that of the apoprotein (27). If wide-spectrum loss of specific cell function occurs in the carcinogenic process, for example, loss of the asialoglycoprotein receptor (28), hepatic tumors should be expected to demonstrate diminished drug-metabolizing capacity. This would result in reduced amounts of P-450 and the enzymes synthesizing their heme component. It is not clear, however, why heme-containing enzymes not involved in xenobiotic metabolism (CAT and TRYPD) would also be decreased, or why MHO should increase. Further, in the rat hepatic nodule and carcinoma, the partial loss of drug-metabolizing capacity is restricted to Phase I enzymes, oxygenases responsible for activation of many carcinogens (1, 9). Phase II activities, such as glucuronosyltransferase and glutathione S-transferase, are elevated in these lesions (1, 9). Taken together, these observations suggest that the partial loss of Phase I activities in hepatic tumors reflects altered heme metabolism and not a general decline in hepatic drug-metabolizing function.

We suggest that the altered pattern of hemoproteins and heme metabolism in hepatic tumors may result from decreased levels of intracellular iron, and that decreased levels of iron may facilitate the expression of receptor for the protein growth factor, transferrin. One phenomenon pointing to iron deficiency in these lesions is their failure to demonstrate stainable iron following iron-loading procedures (29). That iron supplies may actually be insufficient for normal cellular function is suggested by the condition of porphyria in griseofulvin-induced hepatic nodules despite elevated levels of ferrochelatase, the enzyme that converts protoporphyrin to heme by insertion of iron (30, 31).

There is increasing evidence that transferrin functions both as an iron transport molecule and a growth factor (32–34). Whether the 2 functions are separable is less evident and, in tumors, the 2 functions may be closely coupled. In normal cells the number of transferrin receptors is modulated by the iron concentration gradient. Cells that are iron depleted have increased numbers of receptors (35, 36). Receptor number is also increased in cells stimulated to proliferate, presumably because of an increased requirement for iron or other metals (33, 37, 38). Although few data have been forthcoming on hepatic tumors, tumors of other tissue origin demonstrate increased numbers of transferrin receptors. (We have found the number of transferrin receptors in a hepatoma ascites line to be several times greater than that of isolated hepatocytes, data not shown). Assuming that an increased number of transferrin receptors provides tumors with a proliferative advantage, the hepatic tumor's inability to store iron that could down-regulate its transferrin receptors may result in inordinate levels of receptors which promote growth.

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