Serum Epoxide Hydrolase (Preneoplastic Antigen) in Human and Experimental Liver Injury[1]

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Abstract

Reports of an increase in a serum epoxide hydrolase (sEH), immunochemically related to microsomal EH in humans and rats with hepatocellular carcinoma (HCC), suggested its use as a serum marker for this disease. We have now measured sEH levels (as either immunochemically determined content or enzyme activity) in a number of human and experimental models of liver disease. sEH was elevated above the normal range in at least 50% of individuals with HCC, including: 3 of 6 northern Californians; 4 of 7 Koreans with hepatitis B-associated HCC; hepatitis B-associated HCC in woodchucks; and male rats receiving chronic treatment with aflatoxin B_1 or ciprofibrate. sEH was rarely elevated in other forms of chronic liver disease. Only 2 of 9 Koreans with hepatitis B-associated cirrhosis, 1 of 8 carriers, but none with chronic active hepatitis or infection with no apparent liver disease had elevated sEH. In addition, no elevations were found in woodchucks with noncancerous viral hepatitis. In aflatoxin B_1- and M_1-treated rats sEH was not elevated in those with only hyperplastic foci or hepatocellular adenomas, and in two rat initiation-promotion protocols sEH was elevated only in those rats which received the entire set of treatments. sEH was also increased during acute hepatotoxicity in rats treated with CCl_4 or 1,2-dibromo-3-chloropropane. The mechanism of increase in sEH during hepatocarcinogenesis appears to be different from that of other markers of HCC, for in the Korean patients, there was no correlation between sEH concentrations and those of α-fetoprotein or ferritin, nor was there a correlation with α-fetoprotein concentrations in the aflatoxin-treated rats. Furthermore, the increase in sEH does not correlate with induction of microsomal EH in the liver of experimental animals. Studies to date indicate that sEH is selective for HCC and severe hepatonecrotic injury, and may be of some use in the diagnosis of HCC, particularly as a complement to other serum markers.

Introduction

The successful treatment of HCC[7] depends in large part upon the early detection of the disease (1, 2). Serum markers, in particular serum AFP levels, are commonly used for the initial diagnosis of this disease (3, 4). AFP is a major fetal serum protein, and its expression during HCC is believed to be due to depression of fetal genes (3, 4). The use of AFP for the detection of HCC does have some limitations, which include elevations in only 15 to 55% of patients with smaller tumors, other conditions that cause increased hepatocyte replication, testicular or ovarian tumors of yolk sac origin, or occasional gastrointestinal cancers, and pregnant women carrying malformed fetuses (particularly with neural tube defects) (2–7). Therefore, while AFP is one of the best serum tumor markers available, screening for HCC in high-risk populations would benefit from a complementary assay.

In 1975, Okita and Farber (8) detected an antigen which was apparently specific for (pre)neoplastic liver tissue, which they referred to as preneoplastic antigen. Subsequent studies demonstrated that preneoplastic antigen did exist in normal liver tissue but was only detect-

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[7] The abbreviations used are: HCC, hepatocellular carcinoma; AFP, α-fetoprotein; EH, epoxide hydrolase; mEH, microsomal epoxide hydrolase; cEH, cytosolic epoxide hydrolase; sEH, serum epoxide hydrolase; anti-MLmEH, rabbit antibody raised against rhesus monkey liver microsomal epoxide hydrolase; anti-HLmEH, rabbit antibody raised against human liver microsomal epoxide hydrolase; CSO, *cis*-stilbene oxide; cGST, cytosolic glutathione S-transferase; ELISA, enzyme-linked immunosorbent assay; HB_sAg, hepatitis B surface antigen; DBCP, 1,2-dibromo-3-chloropropane.

able in detergent-solubilized microsomes (9). Soon thereafter, at least one protein component of preneoplastic antigen was identified as mEH (10). mEH is an immunochemically distinct epoxide hydrolase which hydrolyzes a number of xenobiotic epoxides or xenobiotics metabolized to epoxides, including many chemical carcinogens (11–13). mEH activity is induced following treatment by a number of diverse xenobiotics (11, 12) and is elevated in preneoplastic and neoplastic rat liver tissue induced by some (14, 15) but not all carcinogens (16). mEH, however, is not induced in human liver tumor tissue (17, 18). mEH differs from AFP by its membranous localization and its lack of specific expression in fetal tissue. Its measurement in serum is therefore a potential complement to AFP, if it is elevated in the blood of humans or experimental animals with HCC.

Based upon substrate specificity, a sensitive radiometric assay was developed by Hammock *et al.* (19) to measure sEH activity. These initial studies demonstrated that sEH activity is elevated in the serum of humans with HCC or acute liver injury (19). Concurrent studies by Griffin and Gengozian (20) demonstrated an increase in protein immunochemically similar to mEH in the serum of rats bearing chemically induced hyperplastic nodules or hepatomas. Subsequent studies have now shown that using antisera specific for rhesus monkey liver mEH, a protein immunochemically similar to mEH in the serum of humans can be detected which correlates well with sEH activity (21).

With the sensitive enzymatic and immunochemical assays now available, we have now extended our studies on sEH levels following acute and chronic liver injury. In humans, more extensive data are presented on the response of sEH in HCC, acute liver injury, and progressive hepatitis B-induced liver injury. In experimental animals, we have studied sEH during progressive stages of hepatitis B-induced and chemically induced HCC and compared it to liver enzyme activities following treatment with carcinogenic, hepatotoxic, and hepatotrophic agents.

Materials and Methods

Assays for Serum Epoxide Hydrolase

sEH in serum was detected by four alternative methods. (*a*) The serum samples collected from patients in northern California were assayed for sEH content by our previously described ELISA which utilizes anti-MLmEH (21). (*b*) Serum samples from Korean patients were analyzed for sEH content in a separate laboratory utilizing the ELISA described by Griffin and Gengozian (20), with substitution of anti-HLmEH. Human liver mEH was purified using modifications of the method of Guengerich *et al.* (22), and the protein appeared homogeneous by reducing, analytical polyacrylamide gel electrophoresis.[8] Rabbit anti-HLmEH was prepared essentially as previously described for the anti-rat counterpart (20). (*c*) The mature rats treated with aflatoxin B_1 and aflatoxin M_1 were assayed using our previously described radiometric thin-layer chromatography enzyme assay (19). (*d*) A modification of this assay, the substrate-saturating enzyme assay, was used for measurement in the serum of all other experimental animals. In brief, diluted serum was incubated with [^{3}H]CSO (final concentration, $1–2 \times 10^{-4}$ M, 60 mCi/mmol); the reaction mixture was extracted with 35 μl of methanol containing 10 mg/ml of CSO and its *meso*-diol and separated on Whatman LK5DF silica gel thin-layer chromatography plates with toulene:*n*-propanol (20:1); and the diol spots were scraped off into liquid scintillation vials for analysis.

Other Enzyme or Serological Assays

Rat liver mEH and mEH-like activity in the cytosol toward CSO, cEH activity toward *trans*-stilbene oxide, and cGST activity toward CSO were determined using our previously described radiometric partition enzyme assays (23). Protein was determined using an automated modification of Bradford's technique (23) with bovine serum albumin as standard. AFP levels were determined in the sera from rats treated with aflatoxin B_1 and M_1 by Dr. Stewart Sell's laboratory, as previously described (24). The diagnostic tests performed on Korean patients were previously described (25, 26). In brief, the test for HB_sAg was performed using Ausyme assay kits (Abbott Laboratories, Abbott Park, IL), for anti-HB_s by Ausab-enzyme immunoassay (Abbott Laboratories), and for serum ferritin by radioimmunoassay (RIANEN kits; NEN, Boston, MA). In woodchucks, the serological tests for hepatitis infection included tests for woodchuck hepatitis surface antigen and anti-woodchuck hepatitis surface antigen by cross-reactivity with HB_sAg (Ausria; Abbott Laboratories) and by immunodiffusion assays, and for anti-woodchuck H_c and anti-woodchuck H_e using the commercially available Corzyme and HB_e (rDNA) kits (Abbott Laboratories), respectively. These assays were performed as described in more detail by Millman *et al.* (27).

Studies on Human Patients

Studies on Northern Californians. Sera used to monitor sEH levels in patients from northern California were aliquots of serum prepared from blood collected for routine diagnosis from the University of California Davis Medical Center at Sacramento. These included sera from 24 apparently normal patients, 6 patients awaiting surgery for diagnosed HCC, and 114 patients with presumed liver disease as indicated by at least one abnormal liver function test. sEH was determined by ELISA using anti-MLmEH.

Studies on Koreans: Involvement of Hepatitis and HCC. Serum samples were collected from 16 male and 8 female patients who attended a liver disease clinic at Seoul National University Hospital Seoul, South Korea, with chronic liver diseases associated with chronic hepatitis B infection. In addition, serum samples were obtained from 9 healthy carriers of hepatitis B, 7 healthy individuals with anti-HBs, and 7 persons without hepatitis B markers. The diagnosis of chronic active hepatitis or cirrhosis was based upon examination of liver biopsies. Patients with HCC were diagnosed by surgery, biopsy, or radiology. Detection of hepatitis markers and diagnosis of liver disease were performed as previously described (25, 26). sEH content was determined in these sera by ELISA with anti-HLmEH.

[8] Griffin *et al.*, unpublished data.

Studies on HCC in Experimental Animals

Woodchucks, Involvement of Hepatitis, and HCC. Sera were provided form wild-caught woodchucks. The woodchucks were trapped in New York, Pennsylvania, or other parts of the middle eastern United States. They were either already infected or developed infections with woodchuck hepatitis virus during captivity. Housing, diagnosis of woodchuck hepatitis virus infection, and morphological detection of chronic active hepatitis, active viral hepatitis, or HCC using serology and liver morphology were performed as described by Millman *et al.* (27).

Experimental Chemical Carcinogenesis. Serum samples, and in some cases liver tissue, were collected from rats undergoing four treatment protocols resulting in different stages of liver cell carcinogenesis. (*a*) Male Fisher 344 rats (Charles River Breeding Laboratories, Wilmington, MA) received dietary treatment with ciprofibrate (0.025% w/w in the diet), for a period of 60 weeks, as described by Rao *et al.* (16). Sera were prepared from blood collected at necropsy and frozen at −70°C until analysis. Histological confirmation of carcinogenesis was performed as previously described (16). (*b*) Male Fisher 344 rats (specific-pathogen-free; Charles River Breeding Laboratories) received dietary treatment with aflatoxin B_1 and aflatoxin M_1, both at 50 ppb in an agar-based semisynthetic diet for 48 to 98 weeks as described by Cullen *et al.* (28). Controls received the same agar-based diet. Sera were prepared from blood collected at necropsy, and diagnosis of stages of carcinogenesis in liver was performed as described (28). (*c*) Infant male Fisher 344 rats were given a single injection of 1 μg/g body weight of aflatoxin B_1 and then given phenobarbital (0.05%) in their drinking water for 16 months. Study groups included infant rats which received both aflatoxin B_1 and phenobarbital, aflatoxin B_1 alone, phenobarbital alone, or vehicle alone. Sera were prepared from blood collected at necropsy, and liver sections were processed as described by Cullen *et al.* (28). (*d*) Using a previously described initiation-promotion regimen (29), male Sprague-Dawley rats were given two-thirds hepatectomy or sham operations while under ether anesthesia. At 24 h, the rats received a single oral dose of diethylnitrosamine (10 mg/kg) or vehicle (5 ml/kg water); and after 8 weeks, selected rats were treated with phenobarbital in their drinking water (0.05%). Rats were then killed at 32 weeks, blood was collected from the abdominal aorta, and livers were perfused with isotonic saline. Homogenates were then prepared for the isolation of microsomal and cytosolic cell fractions as previously described (29). sEH activities for all of the above were determined using the radiometric enzyme assay, and liver enzyme assays were performed as described above.

Experimental Liver Injury. Serum and liver fractions were available from previously described rats with acute hepatotoxic or hepatotrophic changes (29). Briefly, male Sprague-Dawley rats were treated with the following compounds: a single oral dose of CCl_4 (1.0 ml/kg in mineral oil) or DBCP (0.1 ml/kg in mineral oil) and sacrificed at 48 h; three daily i.p. injections of phenobarbital (50 mg/kg in saline) or 3-methylcholanthrene (25 mg/kg in corn oil) and sacrificed at 72 h; or dietary clofibrate (0.5% in ground chow) for 2 weeks. Control animals received the appropriate vehicle. Blood was collected and liver fractions were prepared as previously described (29). sEH activities were determined using the radiometric enzyme assay, and liver enzyme assays were performed as described above.

Statistical Analysis

sEH levels have been presented as population scattergrams to display interindividual variations. Tests for statistical significance were performed in two ways. For experimental groups compared solely to the matched control group, statistical significance was determined using Student's two-tailed *t* test ($P < 0.05$). For experimental groups where there was an apparent progression by diagnostic staging, significant differences within the groups were first determined using one way analysis of variance ($P < 0.05$). If a significant difference existed within the groups, then significant differences between them was determined using the Tukey ranking test ($P < 0.05$) (30).

Results and Discussion

Detection of sEH. sEH has been demonstrated to share substrate specificity with, and to be immunochemically related to, mEH in both rats and humans (19–21). For this reason, both immunochemical and enzymatic methods may be employed to determine sEH levels. The two ELISAs used in this study are easy to perform, can assay a number of samples at a time, do not rely upon an active enzyme, and could theoretically be used on less well-preserved specimens. Due to the lack of immunocross-reactivity of mEH between species (31), studies in nonprimates require additional sources of antibodies and standard antigen, making the enzymatic assays more versatile in the experimental setting. Our initial enzyme assay (19) utilized [^{3}H]CSO with a very high specific radioactivity (15 Ci/mmol) and low substrate concentration. This ensures greater sensitivity as a larger percentage of the radioactive substrate would be converted to product. During the course of these studies, we found that substrate with lower specific radioactivity (60 mCi/mmol) but a 1000-fold higher concentration (apparently substrate-saturating) resulted in a 1000-fold higher activity (compare control rat values in Fig. 4 with those in Figs. 5–7). As the lower specific radioactivity is coupled with higher activity in the modification, both are equally sensitive in the detection of sEH activity. When comparing data between the studies, the ELISAs have been differentiated based on the primary antibody (anti-MLmEH and anti-HLmEH), and the enzyme assays have been differentiated based on substrate concentration (non-substrate-saturating and substrate-saturating).

sEH Levels in Humans with Liver Disease. Previous studies on the response of sEH to liver injury using the non-substrate-saturating enzyme assay demonstrated a control range of sEH activity from undetectable (*i.e.*, <0.1 pmol/h/ml) to 2 pmol/h/ml, sEH activities greater than any of the normal patients in 3 of 8 patients with HCC, and activities ranging from 0.1 to 1.4 pmol/h/ml in 19 patients diagnosed with other forms of cancer with no liver involvement (19). These initial findings along with a coincident report on immunochemically detected elevations in the sera of rats bearing chemically induced hepatomas (20) suggested that sEH may prove to be a useful serum marker for HCC. However, marked in-

creases in sEH with acute liver injury (19) left several unanswered questions concerning the elevation of sEH during the progression of HCC, acute liver injury, and hepatotrophic responses of liver cells to xenobiotics.

The anti-MLmEH ELISA was used to measure sEH content in sera from three patient populations: 24 apparently normal patients; 6 of the previously described patients diagnosed as having HCC; and 95 patients who had elevations of one or more serum assays (total bilirubin, alkaline phosphatase, γ-glutamyl aminotransferase, and serum glutamic-oxaloacetic transaminase) indicative of liver injury (Fig. 1). Sera from these latter patients were also assayed for the following substances: cholesterol; albumin; lactate dehydrogenase; and creatinine phosphate kinase. In sera from 75% of the normal patients, sEH content was below the limit of detection of 1 ng/ml of sEH-like immunoreactive material. The remaining 8 sera had levels ranging from 2 to 70 ng/ml. Three of six sera from patients with HCC had sEH concentrations greater than 100 ng/ml, with the mean value being significantly greater than the mean of the normal patients (Fig. 1). In patients with apparent but nonspecific liver injury, 55% had undetectable levels of sEH, and an additional 25% were less than 100 ng/ml. Of the 18 patients with elevated sEH, 14 were in the same range of that for patients with HCC, and 4 were very high (Fig. 1).

In this latter group sera were selected based solely upon elevations in one or more serum assays, with no other diagnostic basis for liver injury. Furthermore, not all the assays used are selective for hepatocellular necrotic injury. In order to test the relationship between elevated sEH and specific assays for liver injury, the values found for the 18 patients with elevated sEH were tested for correlations with the other assays (Table 1). While fairly high correlations were found between the increases in serum albumin and cholesterol with sEH, there was not a significant correlation with the elevation of either of the two enzymes considered indicative of necrotic liver injury, lactate dehydrogenase, or aspartate amino transferase (Table 1).

In another group of patients, sera were available from a study of Koreans infected with hepatitis B or who had varying stages of chronic liver disease including chronic active hepatitis, cirrhosis, and HCC (25, 26). Sera were assayed for sEH content using the anti-HLmEH ELISA (Fig. 2). With this assay, the limit of detection was also 1 ng/ml, and sEH in apparently normal patients ranged from 1 to 60 ng/ml, similar to the range seen in northern Californians. Except for a single hepatitis B carrier, none of the anti-HB_s-positive, hepatitis B carriers, or patients with chronic active hepatitis had sEH levels above the normal range (Fig. 2). Two of 9 patients with cirrhosis and 4 of 7 with HCC had elevated sEH levels. The only statistically significant difference was the mean sEH level for patients with HCC compared to patients diagnosed as normal, those diagnosed as anti-HB_s-positive, and those with chronic active hepatitis. Ferritin and AFP levels were determined in the same serum samples for 17 of these patients (26). While there were some patients with HCC or chronic liver disease that had elevations in all three markers (Table 2), correlations between the three markers were not significant (sEH *versus* AFP, $r = 0.089$; sEH *versus* ferritin, $r = 0.207$; ferritin *versus* AFP, $r = 0.327$; for $n = 17$, a $r \geq 0.412$ would be required for the correlation to be significantly greater than zero at $P < 0.05$). While this is a small patient population, it is of interest to note that sEH, but not AFP, was elevated in patients with HCC who were not anti-HB_s-positive (Table 2).

These studies in humans demonstrate that in two different populations approximately one-half of the HCC patients had elevated sEH levels. There seems to be a slight elevation during the progression of hepatitis B infection, once liver injury has reached the stage of cirrhosis, but not during acute and even in most cases of chronic hepatitis which are not complicated by cirrhosis

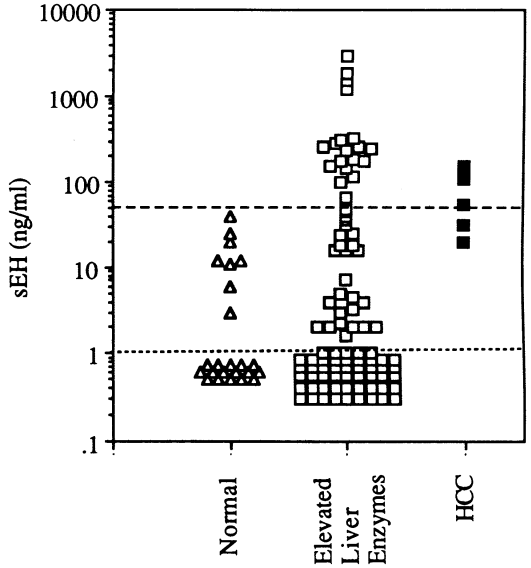

Fig. 1. sEH content in the sera of patients examined in northern California, determined by ELISA using anti-MLmEH. The patient populations were as follows: normal, apparently normal blood donors; elevated liver enzymes, patients with elevated levels of serum glutamic-oxaloacetic transaminase, total bilirubin, alkaline phosphatase, and/or γ-glutamyl aminotransferase; HCC, patients diagnosed with HCC. Each value is the mean of triplicate determinations. – – – –, upper level of normal for this population; · · · · ·, limit of detection for this assay; values below the limit were treated as zero for statistical purposes. The number per group (n) and corresponding mean ± SE were as follows: normal, $n = 24$, 5.4 ± 2.1; elevated liver enzymes, $n = 95$, 114.1 ± 41.4; HCC, $n = 6$, 85.3 ± 23.5 (the last was significantly different from normal as determined by Student's t test; $P < 0.05$).

Table 1 Correlations among serum assays in patients with elevated sEH content

The serum assay values for patients ($n = 18$) with elevated sEH (*i.e.*, >100 ng/ml) were compared by least-squares analysis and the correlation coefficients (r) determined. For $r > 0.400$ the correlation is significantly greater than zero with $P < 0.05$.

Assays being compared		r
Cholesterol	Albumin	0.730
Total bilirubin	Albumin	−0.726
sEH	Cholesterol	0.601
sEH	Albumin	0.586
Total bilirubin	Cholesterol	−0.550
sEH	Alkaline phosphatase	−0.514
γ-Glutamyl aminotransferase	Alkaline phosphatase	0.487
All other comparisons		<0.400

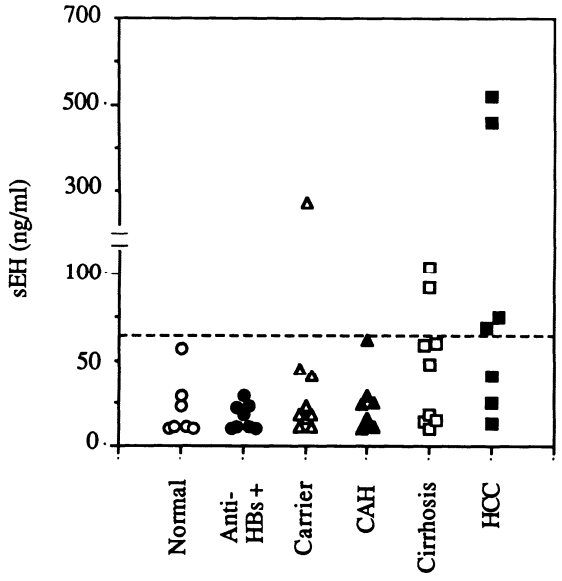

Fig. 2. sEH content in sera of Korean patients determined by ELISA using anti-HLmEH. The patient populations were as follows: normal, apparently normal volunteers; anti-HB_s, otherwise healthy patients with a positive titer for anti-HB_s; carrier, patients diagnosed as being carriers of hepatitis B (*i.e.*, HB_sAg positive with no liver involvement); CAH, patients diagnosed as having chronic active viral hepatitis; cirrhosis, patients diagnosed as having cirrhosis associated with chronic viral hepatitis B infection; HCC, patients diagnosed as having HCC associated with chronic viral hepatitis B infection. The diagnosis of these patients and other tests were described previously (25, 26). Each value is the mean of triplicate determinations. - - - - -, the upper level of normal for this population. The number per group (*n*) and corresponding mean ± SE were as follows: normal, $n = 7$, 11.6 ± 6.6 (*a*); anti-HB_s, $n = 9$, 6.2 ± 2.3 (*a*); carrier, $n = 9$, 40.9 ± 28.3 (*a*, *b*); CAH, $n = 8$, 13.8 ± 6.0 (a); cirrhosis, $n = 9$, 36.7 ± 11.7 (*a*, *b*); HCC, $n = 8$, 178.0 ± 93.3 (*b*). Values for sEH were significantly different as determined by one-way analysis of variance; $P < 0.05$; means which share the same letter in parentheses were not significantly different as determined by the Tukey test, $P < 0.05$).

Table 2 Comparison of sEH content with other indicators of HCC in patients seen in South Korea

sEH levels are the same as shown in Fig. 3 for patients where AFP, ferritin, and HB_sAg levels were available as determined in studies previously described by Hann *et al.* (26).

Diagnosis	Findings in individual patients			
	(nmol/ml)		(ng/ml)	HBsAg
	sEH	AFP	Ferritin	
Chronic active viral hepatitis	14	14	80	+
	52	1	54	+
	3	11	9	+
	6	4	113	+
	15	263	110	+
Cirrhosis	0	20	117	+
	8	0	281	+
	94	0	300	+
	38	188	1420	+
	82	1	10	−
	50	190	780	+
	5	60	192	+
HCC	65	8260	260	+
	573	1079	330	+
	3	0	152	−
	500	4	800	−
	15	20310	920	+

or HCC. In instances of acute necrotic liver injury, even greater elevations of sEH may occur, but sEH is not consistently elevated during hepatonecrotic or other forms of liver injury. Although these studies show that sEH is neither a selective nor a specific marker of HCC, they imply that sEH could be of some use in the diagnosis of HCC. Studies in animal models of liver disease may help clarify some of the limitations of this assay and provide useful models for understanding the mechanism by which sEH is elevated in blood during HCC.

sEH Levels in Animal Models of HCC. The etiology of HCC in humans is most often linked to infection with hepatitis viruses B and C (1, 32). While experimental animal models for induction of HCC by chemicals abound, examples of HCC induced by persistent infection with hepatitis B-like virus are limited to those seen in woodchucks, ground squirrels, and Pekin ducks (27, 33). Sera from wild-caught woodchucks with varying degrees of hepatitis-associated liver injury were analyzed for sEH activity (Fig. 3). The activity in woodchucks with chronic active or acute viral hepatitis was not different from that of hepatitis-free animals. In woodchucks with histologically diagnosed HCC or precancerous lesions, however, sEH activity was elevated beyond the range of the normal animals in 3 of 7 cases, with the mean activity approximately nine times that of hepatitis free animals (Fig. 3). Therefore, in an animal model for virus-induced cancer, changes in sEH levels parallel those found in humans, where viral hepatitis also appears to be a primary carcinogenic agent.

Aflatoxin B_1 is a potent hepatocarcinogen in laboratory animals (34) and in some parts of the world may play a role in the etiology of human HCC (35). Aflatoxin M_1 is a hydroxy metabolite of aflatoxin B_1 which is excreted in the milk of cows and other mammals and therefore is a frequent contaminant of milk destined for human consumption (28). As previously described (28), aflatoxin B_1 (50 ppm) in the diet caused the formation of preneoplastic foci in the livers of all rats by 48–49 weeks, which progressed to carcinomas in approximately 90% of the rats by 77–79 weeks. While a few aflatoxin M_1-treated rats (50 ppm in the diet) had preneoplastic foci and one had hepatocellular adenomas at 77–79 weeks, even at 94–97 weeks no carcinomas had formed (Fig. 4*A*). In the aflatoxin B_1-treated rats, sEH activity was at control levels in rats with only preneoplastic foci (48–49 weeks) but increased significantly in animals with carcinomas (77–79 weeks). AFP levels were significantly elevated in the aflatoxin B_1-treated rats at both time periods (Fig. 4, *B* and *C*). Aflatoxin M_1 treatment did not produce any significant elevations in either sEH activity or AFP content at any time during the treatments (Fig. 4, *B* and *C*). Comparison of the sEH activities and AFP content for individual rats shows that there was no strong correlation between the responses of these two serum assays when comparisons were made for all rats ($r = 0.49$), all treated rats ($r = 0.49$), or rats receiving distinct treatments (B_1 only, $r = 0.41$; M_1 only, $r = 0.10$). While AFP was a more sensitive indicator of a preneoplastic state in the aflatoxin B_1-treated rats, this was not consistent, since rats treated

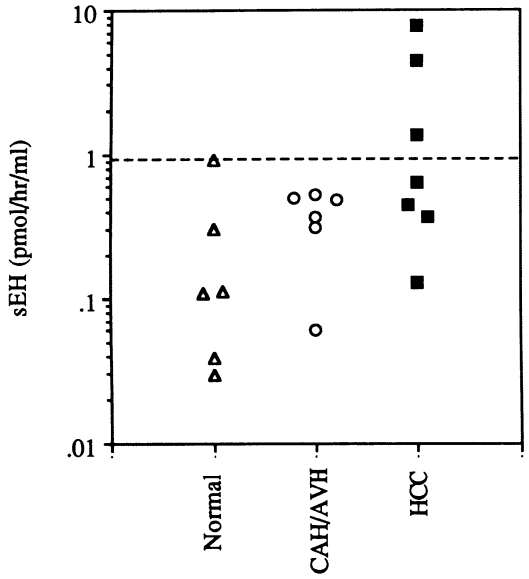

Fig. 3. sEH activity in sera from wild-caught woodchucks determined by substrate-saturating enzyme assay. Sera were prepared from blood collected from wild-caught woodchucks which had been observed in captivity for 6 to 24 months. Diagnoses were as follows: normal, no apparent liver disease or infection with woodchuck hepatitis virus; CAH/AVH, woodchucks which had chronic active or acute viral hepatitis; HCC, woodchucks with histologically diagnosed HCC associated with chronic woodchuck hepatitis viral infection. Each value is the mean of triplicate determinations. - - - - -, upper level of normal for this population. The number per group (n) and corresponding mean ± SE were as follows: normal, $n = 6$, 0.25 ± 0.14; CAH/AVH, $n = 6$, 0.38 ± 0.07; HCC, $n = 7$, 2.17 ± 1.09. No significant difference between mean values was found using one-way analysis of variance; $P < 0.05$.

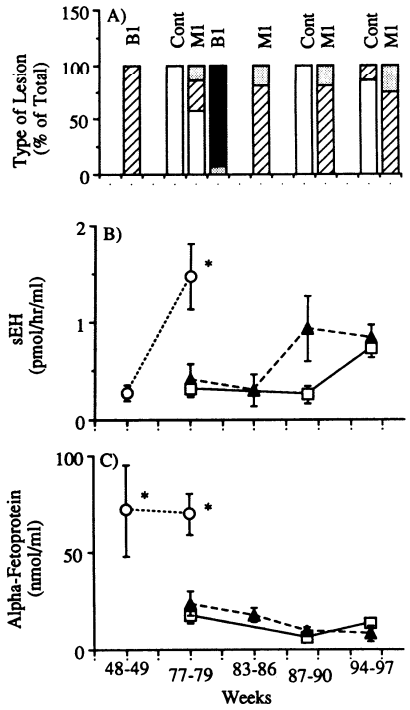

Fig. 4. Involvement of liver with (pre)neoplastic lesions and sEH activities and AFP contents in rats treated with aflatoxin B_1 and aflatoxin M_1. Male rats received dietary treatment with aflatoxin B_1 (○), aflatoxin M_1 (▲), and control (□) diets for the time periods indicated. *A*, summary of involvement of livers of rats with: no (pre)neoplastic lesions (□), hyperplastic foci (▨), hepatocellular adenomas (□), and carcinomas (■). Each cluster of bars represents rats necropsied at the times indicated on the bottom chart. *B*, sEH activity determined by non-substrate-saturating enzyme assay; *C*, AFP levels. A more detailed description of the histological findings has been published previously (28). Values are the mean ± SE for 4 to 10 rats, with each serum assay performed in triplicate. (*, significantly different from matched controls as determined by Student's t test; $P < 0.05$).

with aflatoxin M_1, which had a similar or more extensive involvement of foci or adenoma development, did not have increased levels of AFP. In this model system, sEH activity was elevated only in rats with neoplastic but not preneoplastic lesions.

Peroxisome proliferators are different from many model chemical carcinogens in that they are nongenotoxic (36). Although the morphological features of liver tumors induced by peroxisome proliferators are very similar to those induced by genotoxic carcinogens, in some respects their phenotypic properties are different (36). In sera from rats treated with 0.025% ciprofibrate in the diet for 60 weeks (a protocol which results in the development of HCC in 100% of rats), sEH activity was significantly higher in all five ciprofibrate-treated rats in comparison to matched controls (Fig. 5*A*). It is of interest that peroxisome proliferators, in common with most other hepatocarcinogens, decrease the activity of most cytochrome P450-dependent drug-metabolizing enzymes in neoplastic lesions. Peroxisome proliferators decrease, while most genotoxic hepatocarcinogens increase, mEH and phase II drug-metabolizing enzymes (16). Decreased mEH is also found in human liver tumors (17, 18).

It has been suggested that different parameters can be used in initiation-promotion models to reproduce and dissect specific steps in the development of carcinomas (37). We have now studied sEH activity in two initiation-promotion models (Figs. 5*B* and 6*A*). In the first model, infant male rats were given a single dose of aflatoxin B_1 as the initiator, with phenobarbital provided in the drinking water for 67 weeks as the promoter. When either the initiation or promotion treatment was given alone, there was no increase in sEH activity beyond the range of matched controls. However, in serum from all five rats treated with the complete regimen, sEH activity increased to or beyond the upper limits of the controls (Fig. 5*B*). In a second initiation-promotion regimen, mature male rats underwent a partial hepatectomy, followed at 24 h by a single dose of diethylnitrosamine, and after 8 weeks, promotion was provided by phenobarbital in the drinking water for an additional 24 weeks. Again, none of the incomplete regimens resulted in a significant increase in sEH activity. However, the sEH activity of one of the three rats which had hepatectomy plus diethylnitrosamine was somewhat elevated. Fifty % of the rats (3 of 6) on the complete regimen had elevated sEH activities, with a mean value significantly elevated above that of any of the other treatment groups (Fig. 6*A*).

For these animals, liver EHs and cGST activities were also monitored. cGST activity toward CSO was elevated

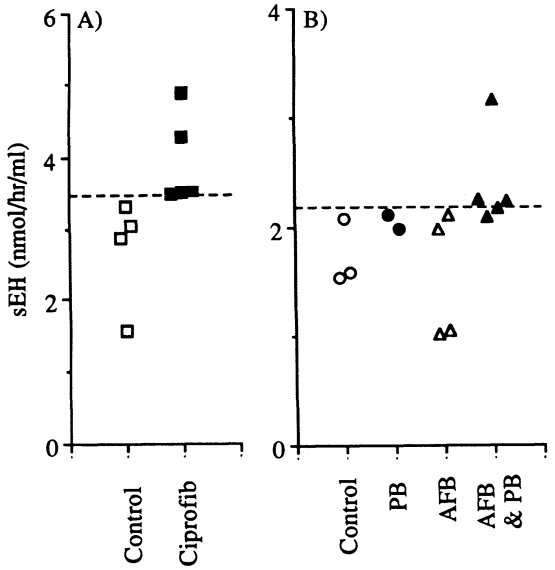

Fig. 5. sEH activities in sera determined by substrate-saturating enzyme assay. *A*, control and ciprofibrate-treated (Ciprofib, 0.025% ciprofibrate in the diet for 60 weeks) male F-344 rats. The number of animals per group (*n*) and corresponding mean ± SE were as follows: control, $n = 4$, 2.70 ± 0.33; Ciprofib, $n = 5$, 3.93 ± 0.25.* *B*, Male F-344 rats received the stated combination of treatments of a single injection of aflatoxin B_1 when infants (AFB) followed by 67 weeks of phenobarbital in their drinking water (PB). The number of animals per group (*n*) and corresponding mean ± SE were as follows: control, $n = 3$, 1.73 ± 0.17; PB, $n = 2$, 2.04 ± 0.07, AFB, $n = 4$, 1.53 ± 0.29; and AFB & PB, $n = 5$, 2.38 ± 0.20.* For both groups, each value is the mean of triplicate determinations. - - - - -, upper level of normal for these populations. (*, significantly different from controls as determined by Student's *t* test; $P < 0.05$).

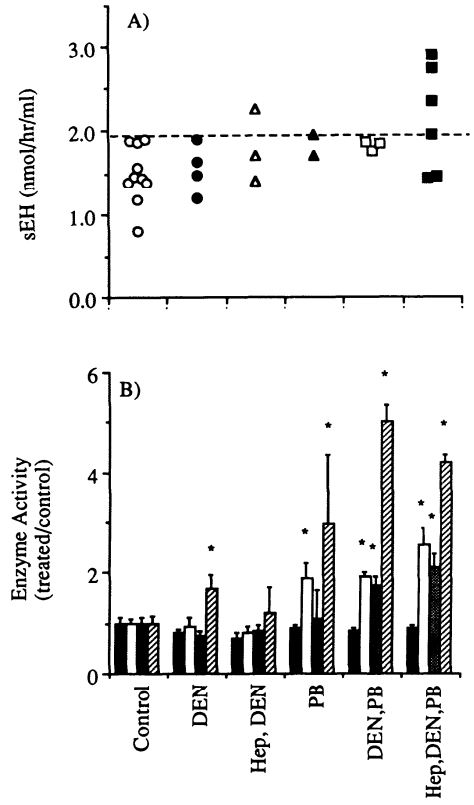

Fig. 6. Comparison of sEH activity with liver EH and GST activities in male rats treated with an initiation-promotion protocol. Male rats received one or a combination of the following treatments: Hep, partial hepatectomy at 0 h; DEN, a single injection of diethylnitrosamine at 24 h; PB, phenobarbital in drinking water from 8 to 32 weeks. *A*, sEH activity for individual rats as determined by substrate-saturating enzyme assay. Each value is the mean of triplicate determinations. - - - - -, upper level of normal for these populations. The number of animals per group (*n*) and the corresponding mean ± SE for sEH activities were as follows: control, $n = 10$, 2.08 ± 0.16; DEN, $n = 3$, 2.17 ± 0.21; Hep, DEN, $n = 3$, 2.50 ± 0.35; PB, $n = 2$, 2.56 ± 0.17; DEN, PB, $n = 3$, 2.55 ± 0.05; Hep, DEN, PB, $n = 6$, 3.00 ± 0.36.* In *B*, liver enzyme assays were performed on microsomal and cytosolic cell fractions for cEH (■), mEH (□), mEH-like activity in the cytosol (□), and cGST activity for CSO (▨). Values are the mean ± SE for the number mentioned above for sEH activity, with each individual value the mean of triplicate determinations. (*, significantly different from controls as determined by Student's *t* test; $P < 0.05$).

by diethylnitrosamine alone, phenobarbital alone, and combinations of the two, with or without prior hepatectomy. mEH activity was elevated, to a lesser extent by phenobarbital alone or any combination of phenobarbital with the initiator diethylnitrosamine. The mEH-like activity in the cytosol was also elevated, but only by the latter two treatment regimens, while none of the treatments had a significant effect on cEH activity (Fig. 6*B*). While there was an apparent relationship between the increase in average activities of mEH, mEH-like activity in cytosol, and cGST activity with that of sEH (Fig. 6*B*), this did not persist when the changes in individual rats were compared. In no case did plots of the increase in tissue activity (relative to the control mean) *versus* the relative increase in sEH activity in the same rat result in a correlation coefficient which was significantly different from zero (data not shown).

Previous experiments with humans and rats have shown that sEH activity can be elevated during some acute hepatonecrotic but not hepatotrophic changes (19, 38). We have measured sEH in rats given a more extensive panel of hepatotoxins and used this as another opportunity to compare serum changes with those in liver cell fractions (Fig. 7). CCl_4 treatment resulted in an extensive and significant increase in sEH activity as previously reported (19). DBCP, which is only mildly hepatotoxic, resulted in a slight but insignificant increase in sEH activity (Fig. 7*A*). In contrast, three treatments which induce mEH activity to varying degrees, phenobarbital, 3-methylcholanthrene, and clofibrate, had no effect on sEH activity (Fig. 7*A*). The lack of association between the induction of the mEH and sEH activities was most clearly seen when the responses to CCl_4 and DBCP were compared. DBCP produced a marked induction of mEH activity, while CCl_4 had no effect (Fig. 7*B*). This was also true when responses in individual rats were compared. Thus the responses of none of the tissue enzyme activities (relative to mean control values) were significantly correlated with the response of sEH activity (data not shown).

Several studies have suggested that an increase in mEH (preneoplastic antigen) activity or content may be an appropriate marker from preneoplastic and neoplastic lesions in the liver (8, 14, 15). However, this is not

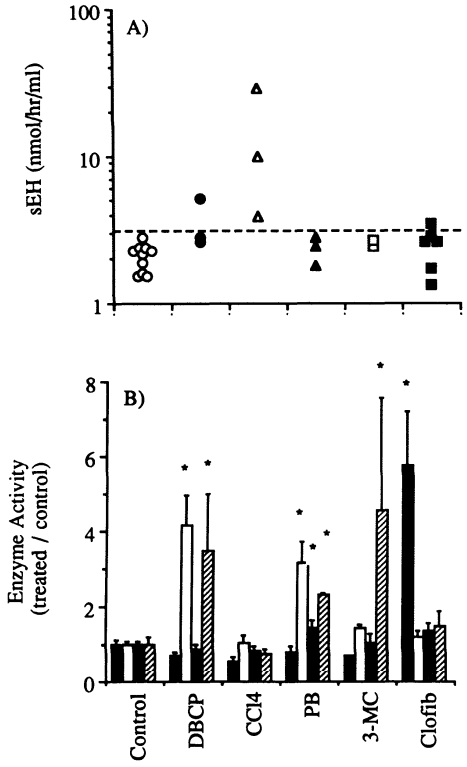

Fig. 7. Comparison of sEH activity with liver EH and GST activities. Procedures were as follows: single intragastric treatment of DBCP or CCl_4 for 48 h; PB, 3 daily injections of phenobarbital; 3-MC, 3 daily injections of 3-methylcholanthrene; Clofib, dietary administration of clofibrate for 2 weeks. *A*, sEH activity for individual rats as determined by substrate-saturating enzyme assay. Each value is the mean of triplicate determinations. - - - - -, upper level of normal for these populations. The number of animals per group (*n*) and the corresponding mean ± SE for sEH activities were as follows: controls, $n = 10$, 2.08 ± 0.14; DBCP, $n = 3$, 3.53 ± 0.80*; CCl_4 for 48 h, $n = 3$, 14.14 ± 7.50*; PB, $n = 2$, 2.33 ± 0.29; 3-MC, $n = 2$, 2.53 ± 0.12; Clofib, $n = 6$, 2.43 ± 0.32. In *B*, liver enzyme assays were performed on microsomal and cytosolic cell fractions for cEH (■), mEH (□), mEH-like activity in the cytosol (□), and GST activity for CSO in the cytosol (▨). Values are the mean ± SE for the number mentioned above for sEH activity, with each individual value the mean of triplicate determinations. (*, significantly different from controls as determined by Student's *t* test; $P < 0.05$).

consistently true, particularly for nongenotoxic hepatocarcinogens such as the peroxisome proliferators and ethionine (16, 39). Furthermore, an increase in mEH activity in human neoplastic and preneoplastic lesions is not seen (17, 18). In previous studies (19, 20) and the current report, we have shown that sEH activity or content is elevated in a majority of the individual humans or experimental animals with HCC. The latter have included classic models of genotoxic carcinogenesis, initiation by genotoxins followed by promotion, models of peroxisome proliferator nongenotoxic carcinogenesis, and viral hepatitis-induced HCC. Additional experiments in rats treated with hepatotoxins have further demonstrated that the increase in sEH is not dependent upon a concomitant increase in liver mEH.

The association of increased sEH with liver cancer and acute liver injury suggests that the liver may be the origin of sEH; mechanisms other than excess production during induction of mEH must be explored to understand why serum levels of this enzyme increase. Griffin and Kizer (9) have demonstrated that in microsomes from liver (pre)neoplastic lesions, the mEH (preneoplastic antigen) differs from that in normal microsomes by its ability to be detected during immunodiffusion without the prior disruption of the membranes by detergents. Furthermore, it has been shown that in these altered microsomes, mEH is readily released from the microsomes during incubation, and that a concomitant increase in mEH activity occurs in liver cytosol (14). In a subsequent report, studies from the same laboratory indicated that the release of sEH into the blood was correlated with the size of chemically induced hyperplastic lesions and that incubated slices of the lesions would release sEH into culture media. However, the rate of release was not correlated with the extent of development of the lesion (40). Increased mEH-like activity in the cytosol from neoplastic lesions from human livers has also been observed (17). Therefore, the shedding of mEH from altered membranes, with subsequent release into the bloodstream, offers a possible mechanism for increases in sEH. However, increases in mEH-like activity in liver cytosol have also been observed to be coincident with the induction of mEH by noncarcinogens (39), and as observed in this study, the increase in cytosolic mEH-like activity does not correlate with increases in sEH. As sEH increases during acute liver cell necrosis, the possibility that the increase during carcinogenesis also reflects the presence of necrotic cells in the neoplastic lesions must be considered. Indeed, it may require a combination of disruption of hepatocyte plasma membranes and alteration of the endoplasmic reticulum to increase sEH.

While the results offered in this study provide only suggestive evidence for the latter mechanism for the release of sEH, they do demonstrate that sEH is increased in a majority of individual humans and experimental animals during hepatocarcinogenesis. The limited data available also suggest that sEH is not increased by other forms of cancer. Although it does appear to increase during acute necrotic injury to the liver, this occurs with AFP too, and necrosis can usually be clinically differentiated from HCC. Furthermore, preliminary evidence suggests that the increases in sEH do not always correlate with those seen with AFP. Together, these data provide the motivation for pursuing more thorough studies on the utilization of sEH assays as a complementary screen to AFP for the diagnosis of HCC.

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