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Sequential Changes in Histochemical Localization and Releasability of Epoxide Hydrolase during Experimental Hepatocarcinogenesis

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Abstract The influence of chronic 2-acetylaminofluorene (2-AAF) feeding on the histochemical localization of epoxide hydrolase in liver tissue was studied. Epoxide hydrolase was detected in the cytosolic fraction and the incubated microsomal supernatant by electroimmunoassay during 2-AAF feeding. A general increase in hepatocyte staining was observed from week 1 to week 3 during the feeding of 2-AAF. Early foci, *i.e.* week 6, showed low or slight staining, while hepatocytes surrounding them were stained intensely. In contrast, in late hyperplastic nodules, epoxide hydrolase was localized only in nodular cells. There quite a difference between the foci and the late hyperplastic nodules. A histochemical study of epoxide hydrolase indicated a correlation between the intensity of staining and the amount of epoxide hydrolase in cytosol or releasability from incubated microsomes.

Key Words: Hepatocarcinogenesis-2-Acetylaminofluorene-Epoxide hydrolase-Hyperplastic nodule-Rat

Introduction

It is believed that most hepatocarcinogens require metabolic activation in the liver[30] and that enzymes located in microsomal membranes play a prominent role in the activation process[17,29]. Many of the histological, biological and histochemical changes that are observed in precancerous hepatic tissues are accompanied by changes in the properties of microsomal membranes[1,19,21]. Drug-metabolizing enzymes which are localized in these membranes may be a primary target for activated hepatocarcinogens. Alterations of membrane enzymes following their interactions with hepatocarcinogens may produce physiological perturbations that eventuate full malignancy[30].

Epoxide hydrolase, one of the important detoxification enzymes localized in the ER membrane, hydrolyzes reactive epoxides to dihydrodiols[16,20]. Previously, we reported

The abbreviation used are: ER, endoplasmic reticulum; TKMS, 250mM sucrose, 24mM KCl, 5mM $MgCl_2$, 50mM Tris· HCl, pH 7.6; PAP, peroxidase anti-peroxidase; FITC, fluorescein isothiocyanate; γ-GTP, gamma-glutamyl transpeptidase.

that epoxide hydrolase, a preneoplastic antigen, was released from the microsomal membranes of hyperplastic nodules and hepatomas induced by 2-AAF[7,8].

In the present paper, by using rat livers obtained during progressive stages of carcinogenesis induced by 2-AAF, we studied the quantitative and qualitative changes of epoxide hydrolase released from the microsomal membranes into cytosolic fractions and incubated microsomal supernatant fractions. By using histochemical staining of epoxide hydrolase, we also correlated cellular enzyme localization with the release phenomenon.

Experimental Material and Method

Holtzman male rats weighing from 100 to 150 g were obtained from a breeding colony maintained at the Noble Foundation. Throughout 17 weeks of an intermittent feeding schedule, the rats were fed a 0.05% 2-AAF diet for 3 weeks, then a basal diet[16] for 1 week, returned to a 2-AAF diet for 2 weeks, 2 weeks of a basal diet, 3 weeks of a 2-AAF diet, 4 weeks of a basal diet and finally 2 weeks of a 2-AAF diet. Control rats were fed a basal diet only. Six rats were killed at weeks 1, 2, 3, 4, 6, 9, 11, 15 and 17 after the 2-AAF feeding was started, and liver tissues were isolated. Large hyperplastic nodules were induced and isolated after 16 weeks using the same regimen. Hepatomas were diagnosed by laparotomy and histologica examination, and resected at 33 to 45 weeks after intermittent feeding of 2-AAF.

Liver fractionation and preparation of cytosols and incubated microsomal supernatants were performed by the methods previously reported.[8] Microsomes were incubated at 37° for 2 hr with a protease inhibitor, *para*-toluenesulfonyl fluoride (0.5mM) and dithiothreitol (1mM) in a TKMS buffer. After incubation, samples were centrifuged at 110,000g for 2 hr. Supernatant and pellet fractions were obtained and analyed as previously reported.[8]

Epoxide hydrolase was purified from control rat livers by the modified method of Lu *et al.*[15] This preparation gave a single band at 50,000 daltons when analyzed by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis. Following acid hydrolysis an amino acid analysis indicated a composition similar to that reported previously.[14] The styrene oxide hydrolase activity of this preparation was 550-600 nmol/min/mg protein.[8,14,20]

Antiserum was prepared by injecting a rabbit 4 times with 200 μg of purified epoxide hydrolase at two-week intervals. The rabbit was bled once after the last injection. Electroimmunoassay was performed in 1% agarose with 0.2% sodium deoxycholate, 2.4% specific antiserum and a barbital buffer (pH 8.6) using constant voltage electrophoresis (100V for 12hr). The precipitin line was examined by indirect light after the removal of excess protein by successive saline and water washes. In every determination, purified epoxide hydrolase was used as the standard. Protein was determined by the method of Lowry *et al.*[13]

The localization of epoxide hydrolase in liver tissue was estimated by direct immunofluorescent and indirect PAP staining. Liver tissues were fixed in ice-cold 95% ethanol for 24 hr and then in −20° with 100% ethanol for 24 hr. The fixed tissue was embedded in soft paraffin (British Drug House, Poole, 48° melting point) and 4 μ sections were prepared. An IgG fraction of rabbit anti-epoxide hydrolase serum was conjugated with FITC (Bartimore Biological Lab., Cokeysville) by the technique of Kawamura.[11] PAP staining was performed by the method of Nakae *et al.*[19] using peroxidase conjugated anti-rabbit IgG serum (Behring Institute, Marburg) as a secondary antibody. For each tissue block consecutive tissue sections were stained with γ-GTP and hematoxylin-eosin for changes in histological morphology. The localization of γ-GTP was estimated by the method of Rutenburg *et al.*[24]

Results

Qualitative and Quantitative Changes in Epoxide Hydrolase

An immunological similarity of epoxide hydrolases from control microsomes, nodular microsomes and nodular cytosols was detected by double immunoprecipitation analysis (Fig. 1). A single immunoprecipitin band was identical to purified microsomal epoxide hydrolase.

Microsomal epoxide hydrolase content of livers obtained during intermittent feeding

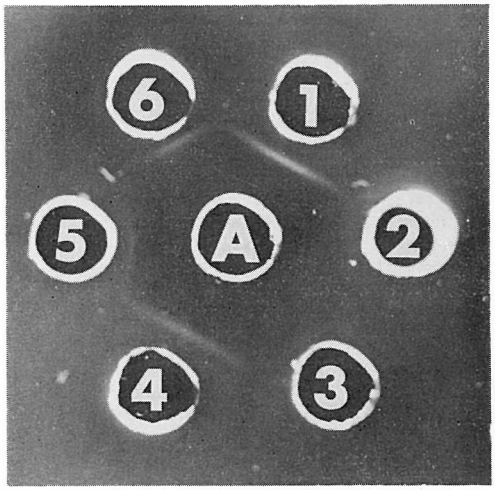

Fig. 1 Double immunodiffusion.
Purified microsomal epoxide hydrolase (well 1 and 4), microsomes (well 2) and cytosols (well 3) of control rat liver, microsomes (well 5) and cytosols (well 6) of hyperplastic nodules and rabbit antiserum to purifiedr at liver microsomal epoxide hydrolase (center well A).

of 2-AAF, and of hyperplastic nodules and hepatomas was quantitated by electroimmunoassay (Fig. 2). The total microsomal epoxide hydrolase protein content increased two fold over control liver microsomes after one week of 2-AAF feeding, and remained essentially constant over the next 17 weeks. The microsomal hydrolase in the hyperplastic nodule was 2.5 times control levels, whereas the microsomal hydrolase in the hepatoma varied from near control levels to greater than six fold the control. The amount of epoxide hydrolase protein in the livers of rats fed the basal diet throughout the 17 weeks remained essentially constant.

The releasability of epoxide hydrolase from microsomes during incubation *in vivo* is also shown in Fig. 2. Two phases of increased epoxide hydrolase releasability from micrsomal membranes were observed. The early phase occurred during weeks 1 through 6 of 2-AAF feeding, while the second phase occurred at 11 weeks and persisted into the hyperplastic nodule and hepatoma stages. We were unable to detect any release of epoxide hydrolase during the incubation of microsomes from control rats. As shown in Fig. 3, the cytosolic fraction of the liver homogenates from rats fed 2-AAF seemed to vary in much the same manner as the amount of epoxide hydrolase released from incubated microsomes (Fig. 2). Cytosol from rats maintained on a basal diet was tested for the presence of epoxide hydrolase protein, but none was detected. Our method readily detected epoxide hydrolase contents as low as 0.1% in the cytosolic fraction.

Changes of Histochemical Localization of Epoxide Hydrolase in Liver Tissue.

Immunofluorescent and PAP staining of sections from control rats showed epoxide hydrolase in hepatocytes distributed throughout the lobular area. Among rats fed 2-AAF ,we detected increased epoxide hydrolase in hepatocytes of the periportal area at 3 weeks ,but almost no epoxide hydrolase in the proliferated ductal cells or connective tissues .At 6 weeks, early foci showed low or slight specificity of epoxide hydrolase, while hepatocytes surrounding foci were stained intensely (Fig. 4 and 5a). Epoxide hydrolase was absent from nuclei but was distributed quite homogenously throughout the cytoplasm (Fig .4). When consecutive tissue sections were analyzed for γ-GTP histochemistry, the focal areas stained more intensively than the surrounding areas (Fig. 5b). The overall intensity of epoxide hydrolase markedly decreased at 9 weeks, and it was difficult to identify nodular localization in these tissue sections. At 11 weeks, epoxide hydrolase was intense in hepatocytes surrounding foci but was less intense in large nodular cells. At 17 weeks, intense epoxide hydrolase was highly localized in nodular cells of almost every hyperplastic nodule (Fig. 6a). The major part of such nodules were almost all positive for γ-GTP histochemical stain (Fig.

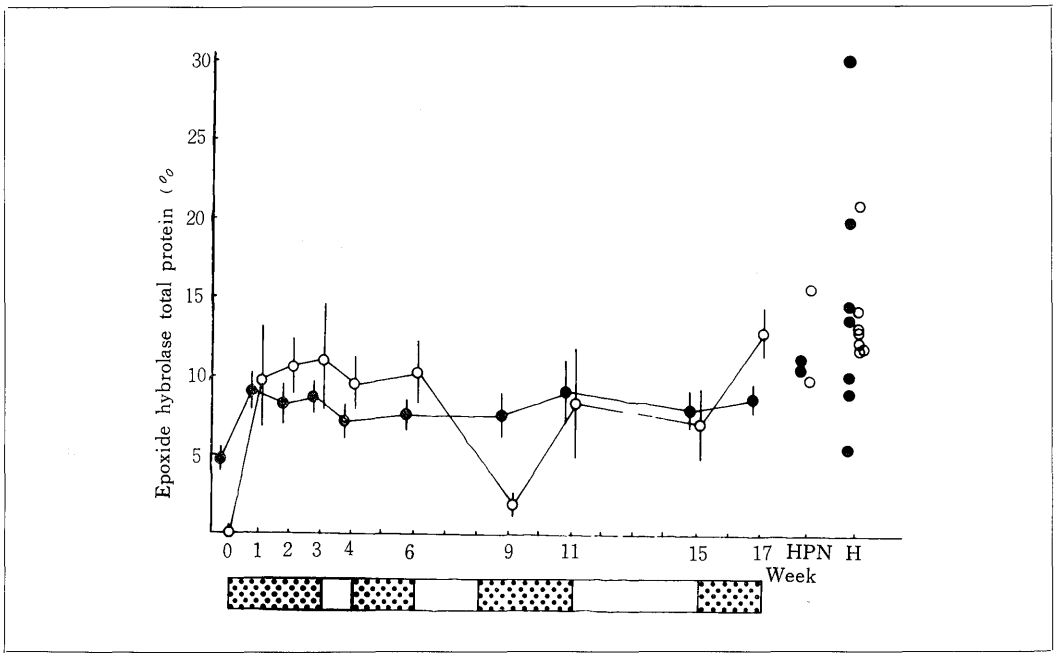

Fig. 2 Sequential changes of epoxide hydrolase content assayed by electroimmunoassay.
Animals were fed a basal diet (open bar) or a basal diet containing 0.05% 2-AAF (strippled bar) for the time periods indicated.
HPN and H stand for hyperplastic nodules and hepatoma respectively. The microsomal enzyme content (closed dots) is expressed as a percentage of the total microsomal protein, and the incubated microsomal supernatant enzyme content (open circles) is expressed as a percentage of the total microsomal incubated supernatant protein. Points with vertical bars represent mean±standard deviation from the data obtained with six rat livers.

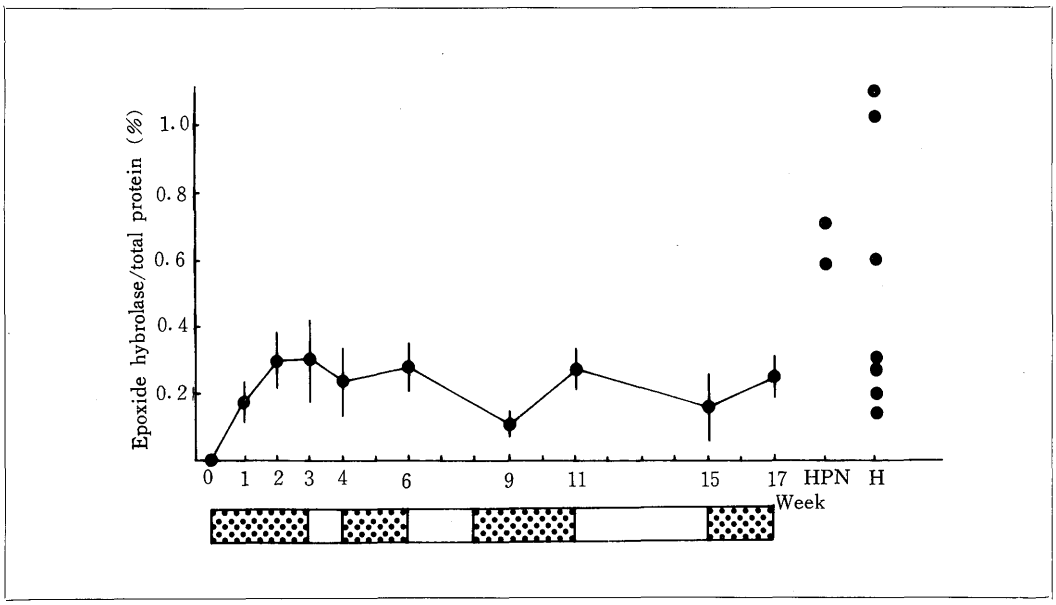

Fig. 3 Sequential changes of epoxide hydrolase content of cytosolic fractions.
The feeding schedule and the number of aminals was the same as in Fig. 2.

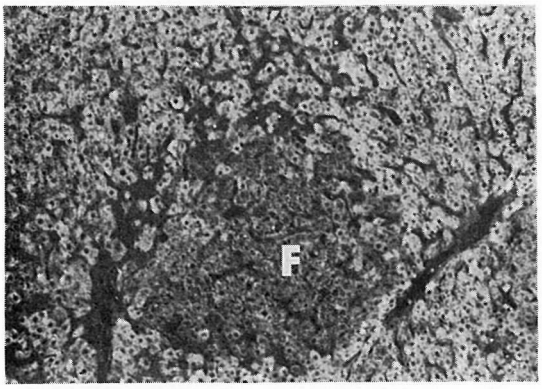

Fig. 4 The localization of epoxide hydrolase in rat liver at week 6 of intermittent 2-AAF feeding.
Early foci showed low or slight specific fluorescence, while hepatocytes surrounding foci were stained intensely. F: early focus. ×160.

6b).

Discussion

Epoxide hydrolase is drug metabolizing enzymes concerned with hydrolating electrophilic epoxide[20]. But still, the physiological role of epoxide hydrolase in early foci, hyperplastic nodules or hepatomas is an unresolved problem. This study was performed to elucidate the changes of epoxide hydrolase during chemical hepatocarcinogenesis, which is one of the major proteins induced by various carcinogens in microsomal membranes[7,8].

Two epoxide hydrolases have been described for rat livers[9]. The epoxide hydrolase under study in the present report is normally located in the ER membrane[19]. The second epoxide hydrolase, which is located primarily in cytosol, is immunologically distinct from the enzyme in the ER membrane[9]. A portion of microsomal epoxide hydrolase is buried in the hydrophobic lipid of ER[25]. In microsomes of control rat livers, about 4% of the total protein was composed of epoxide hydrolase as determined by electroimmunoassay, but no microsomal epoxide hydrolase was detected in cytosols or in incubated microsomal supernatant fractions[8]. The proliferation of smooth ER and the detachment of ribosomes during hepatocarcinogenesis are universal findings *in vivo*[30]. In control microsomal membranes, no epoxide hydrolase was released during homoginization or incubation.

Biological, histological and biochemical changes of rat livers induced by intermittent 2-AAF feeding have been the subject of many investigators. These studies[4,6,23,31] suggest a sequential progression of initiated foci (or small nodules) to large, permanent hyperplastic nodules. It has been postulated that these nodules are precursors of hepatomas. In the present study, histochemical staining of epoxide hydrolase decreased if the foci were compared with the surrounding hepatocytes, although the magnitude of decrease was different among early foci which were intensely stained for γ-GTP. Early proliferative focal cells are islands of cells with altered enzymes[4,11,12,22]. These

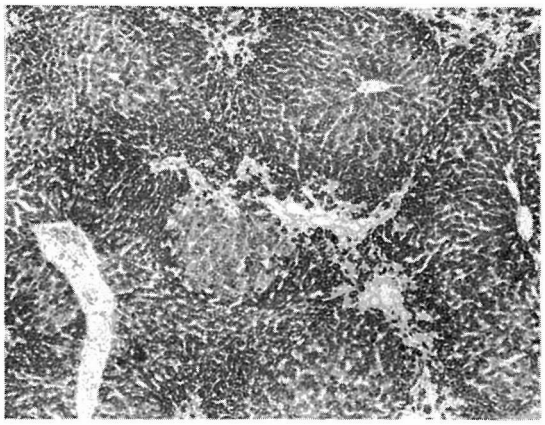

Fig. 5a The localization of epoxide hydrolase in rat liver at week 6 of intermittent 2-AAF feeding. Peroxidase anti-peroxidase staining. ×40.

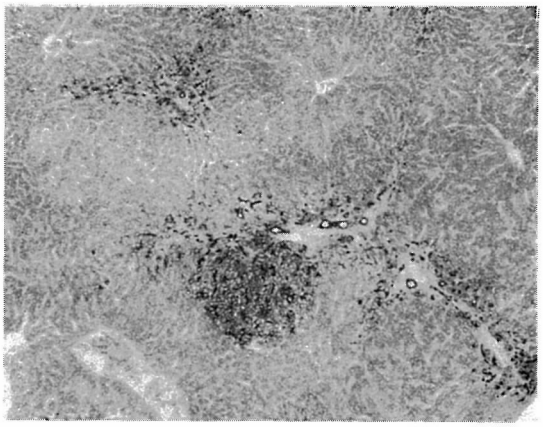

Fig. 5b The localization of gamma-glutamyl transpeptidase in the consecutive tissue section of Fig. 5a. ×40.

initiated hepatocytes show a pronounced resistence to liver toxins[5]. This characteristic may result from a deficiency of several enzymes which are necessary to activate carcinogens and cytotoxins to the active electrophiles[2,21]. In liver tissue with these foci, a toxic effect of carcinogens might induce large amounts of epoxide hydrolase in the surrounding hepatocytes. Through the phase of maturation[12] or regression[28] of hyperplastic nodules, epoxide hydrolase and γ-GTP were stained intensely in late hyperplastic nodules and hepatomas. Presumably, some new properties will be clarified about epoxide hydrolase between surrounding hepatocytes and hyperplastic nodular cells with furth-

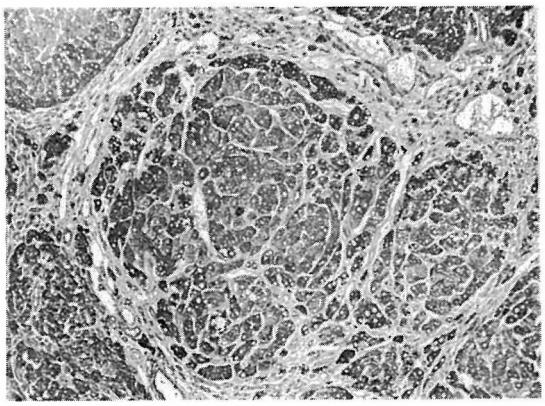

Fig. 6a The localization of epoxide hydrolase in rat liver at week 17 of intermittent 2-AAF feeding. Marked increase of epoxide hydrolase was localized in nodular cells of almost every hyperplastic nodule. Peroxidase anti-peroxidase staining. ×100.

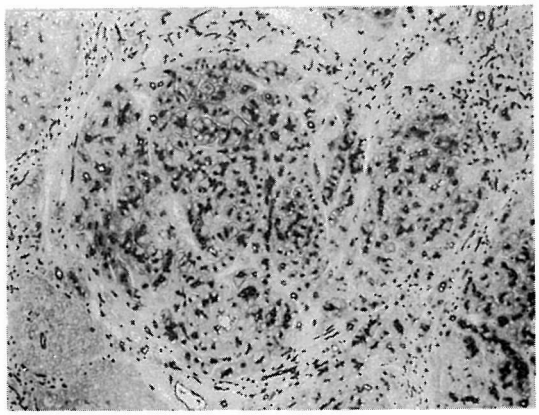

Fig. 6b The localization of gamma-glutamyl transpeptidase in the consecutive section of Fig. 6a. ×100.

er testing[8].

At 9 weeks, in spite of a 1 week 2-AAF feeding, the releasability of epoxide hydrolase markedly decreased. This period coincided with the time when the localization of epoxide hydrolase in liver tissue decreased. At the phase of maturation, some important changes of membranous properties might occur as a phenotypic change in hepatocarcinogenesis. In a later stage of nodular formation, the epoxide hydrolase releasing phenomenon persisted even after cessation of 2-AAF feeding. In the present study, we feel that the reflection of intermittent carcinogen feeding must be considered. But these results corroborate recent studies[3]

using a initiation-selection system for hepatocarcinogenesis[26]. It seems that the membrane alteration induced by 2-AAF has a more permanent physiologic stability in late hyperplastic nodules when compared to the early nodular area, and that the phenomenon underlying these changes about microsomal membranes may be causally and/or coincidentally related to hepatocarcinogenesis.

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