Vol. 98, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 30, 1981 Pages 417-423

FOCAL ELEVATION OF LIVER MICROSOMAL EPOXIDE HYDROLASE IN EARLY PRENEOPLASTIC STAGES AND ITS BEHAVIOUR IN THE FURTHER COURSE OF HEPATOCARCINOGENESIS

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Received December 1,1980

SUMMARY: Treatment of rats with N-nitrosomorpholine (NNM) for 7 weeks led to a focal increase in liver microsomal epoxide hydrolase (EH) as early as 2 weeks after withdrawal of the carcinogen. This treatment also leads to hyperplastic nodules and liver tumors, but much later. At the same early time point, ATPase activity was decreased in the same islands. Most of these areas already had increased γ-glutamyltranspeptidase activity. The increase in EH at this early time point was more distinct than the decrease in ATPase which has thus far been considered a suitable marker of the earliest stages in hepatocarcinogenesis. The focal increase in EH was also observed in all benign hepatomas, but not in any of the hepatocellular carcinomas investigated so far.

INTRODUCTION: Many xenobiotics of diverse chemical structure (1) including hepatocarcinogens (2-4) induce the microsomal epoxide hydrolase (EH). When treatment by the inducing agent is discontinued EH levels drop to normal values (2). Localisation studies in liver sections showed that treatment of rats with 2-acetylaminofluorene results in an increase in the amount of EH protein, preferentially in the periportal area, in a diffuse manner (5).

Contrary to the reversible and diffuse elevation of EH levels right after treatment with the compounds mentioned above, hepatocarcinogens led to a focal and persistent increase in EH activity in distinct hyperplastic

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nodules (2), which were produced according to a treatment schedule developed by Farber and his associates (6). Such focally elevated EH protein was shown to be identical (2) to an earlier described (7) preneoplastic antigen.

In the present study we investigated whether these focal and irreversible alterations of EH appear already in much earlier preneoplastic stages and whether they persist in benign and malignant liver tumors.

MATERIALS AND METHODS:

Animals and administration of carcinogens. Inbred female Lewis rats 8 weeks of age were treated with 10 - 20 mg/kg body weight of the hepatocarcinogen NNM given in drinking water. After the periods of NNM administration stated in the figure legends, feeding was routinely stopped for 2 weeks, and where indicated, for longer periods up to 26 weeks before the animals were killed. The livers were perfused and excised. A part of each liver was frozen immediately at -70°C in isopentane; the rest was fixed as described later.

Purification of EH and immunological procedures. Microsomal EH was isolated from rat liver and purified (8) to apparent homogeneity (8,9). Antibodies against it were produced in rabbits (10) and the IgG fraction isolated by chromatographing the serum over DEAE-Affigel blue (Bio Rad Laboratories, Richmond, CA, USA) and subsequent precipitation of the IgG fraction by the addition of ammonium sulfate to 50% saturation.

Cellular EH was detected by an indirect staining method in which rabbit anti-EH immunoglobulins and enzyme-labelled sheep anti-rabbit IgG antibodies were used. IgG molecules of the "Sandwich" antibodies were conjugated with horseradish peroxidase (HRP, RZ 3) and purified on a Sephadex G 200 column (11).

Enzyme histochemistry. From each liver lobe, serial sections of 6 µm were prepared at -15°C on a cryostat microtome. The first two sections were stained for ATPase and γ-glutamyltranspeptidase (γ-GT) activity, the following two for the immunohistochemical demonstration of EH and α-fetoprotein (AFP), and the last two were stained with toluidene blue and hematoxylin-eosin. 3 - 6 series of each liver were investigated. ATPase activity was demonstrated by the method of Wachstein et al. (12), γ-GT according to Lojda et al. (13) using γ-glutamyl-1-naphthylamide as substrate and p-rosaniline as a coupling agent. The slides were counterstained with hemalaun.

Immunohistochemistry. Cryostat sections were mounted on albumin-coated slides, air-dried and fixed at 4°C for 5 min in PBS containing 6% formaldehyde (from paraformaldehyde) plus 0.1% glutaraldehyde followed by three successive PBS washings of 5 min each. Alternatively, liver slices about 0.5 cm thickness were fixed in 99% ethanol/1% acetic acid for 12 - 15 h at 0°C, dehydrated and embedded in paraffin (14). 5 - 7 µm thick sections were mounted on acetone-cleaned slides, deparaffinated in xylene and passed from absolute ethanol into PBS.

Prior to incubation with antibodies, endogenous peroxidases were irreversibly inhibited by treatment with 1% H_2O_2 in PBS for 90 min. Slides were washed for 5 min in PBS supplemented with 1% bovine serum albumin (BSA) and 0.5 M NaCl (15), then incubated: first, with unlabelled anti-EH

immunoglobulin (0.01 mg/ml) for 24 h at 4°C, second, with horse radish peroxidase (HRP)-labelled anti-rabbit IgG antibodies (0.1 mg/ml) for 20 min at laboratory temperature. Immunoglobulins and antibodies that did not react were removed by three successive washings of 5 min each in 1% BSA and 015 M NaCl-supplemented PBS.

Peroxidase activity was detected as described (16). Sections were treated for 1 min with 0.1% OsO_4/PBS, dehydrated, and mounted under cover slips.

Immunocytochemical specificity was checked by incubation with (a) normal rabbit IgG (0.01 mg/ml) and (b) rabbit anti-EH absorbed with rat liver microsomes. Both procedures were followed by addition of HRP conjugates of sheep anti-rabbit IgG antibodies and enzyme substrate. Details of the methodology of immunoperoxidase cytochemistry have been described (15). For routine histology, sections were stained by hematoxylin-eosin.

RESULTS AND DISCUSSION:

Preneoplastic lesions. During treatment of rats with hepatocarcinogens a constant sequence of distinct cytochemical and histological alteration is found in the liver (17). With respect to the induction time of these preneoplastic alterations the same dose-time relationship holds as for the induction of the liver tumors which appear later on (18).

Figure 1 shows that 94 days after the beginning of NNM treatment according to the schedule described in Methods, EH is clearly increased in distinct liver cell islets. Comparison of Figure 1A and 1B shows that the identical areas are deficient in ATPase. This exact spatial coincidence was observed in all sections investigated. At the earliest time point investigated (64 days) the increase in EH could be easily seen, while the decrease in ATPase was less readily detectable. Therefore, EH appears to be more suitable than ATPase as a marker for the earliest stages of hepatocarcinogenesis. γ-GT, which is also a positive marker, was consistently detectable in the EH/ATPase-altered areas not at these early (64 days) but only at much later time points (Figure 1C).

In order to see whether the increase in EH was reversible, livers were investigated at 2, 6, 12, 18 and 26 weeks after discontinuation of NNM feeding. The elevation of EH persisted throughout the entire observation period. The same was true for ATPase deficiency and γ-GT elevation.

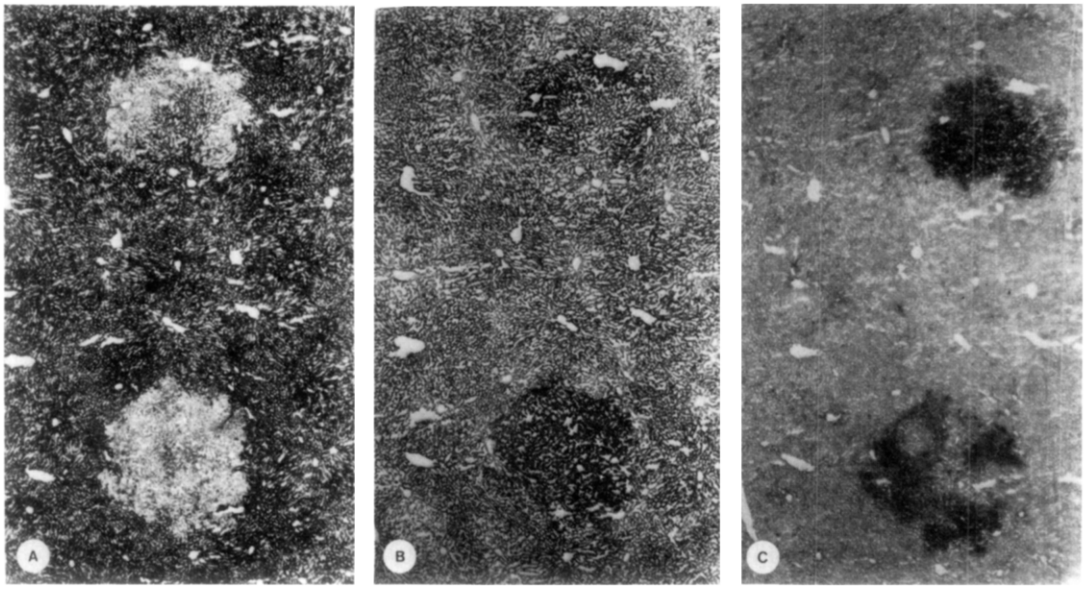

Fig. 1. Parallel sections obtained from the liver of a rat 94 days after beginning of treatment with 10 mg/kg NNM. (A) ATPase reaction; (B) Immunohistochemically stained for microsomal EH; (C) γ-GT reaction.

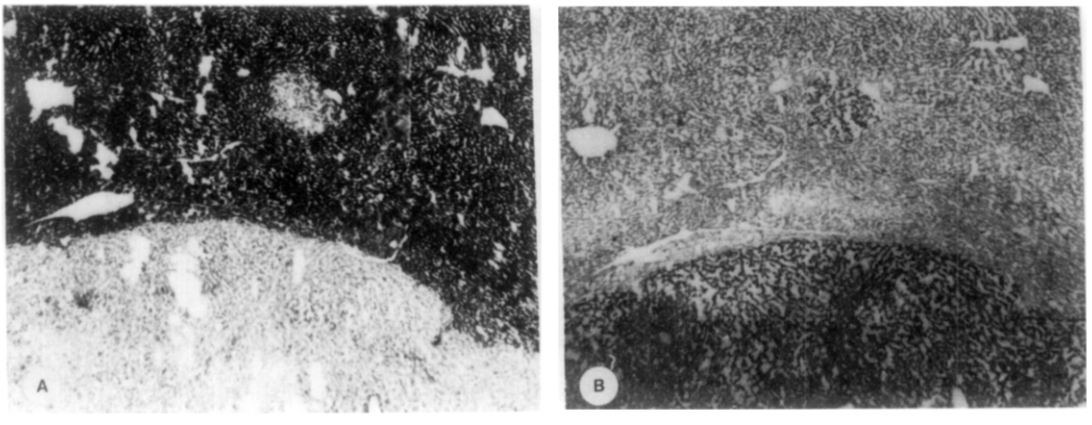

Fig. 2. Parallel sections obtained from the liver of a rat 150 days after beginning of treatment with 10 mg/kg NNM. (A) ATPase reaction; (B) Immunohistochemically stained for microsomal EH.

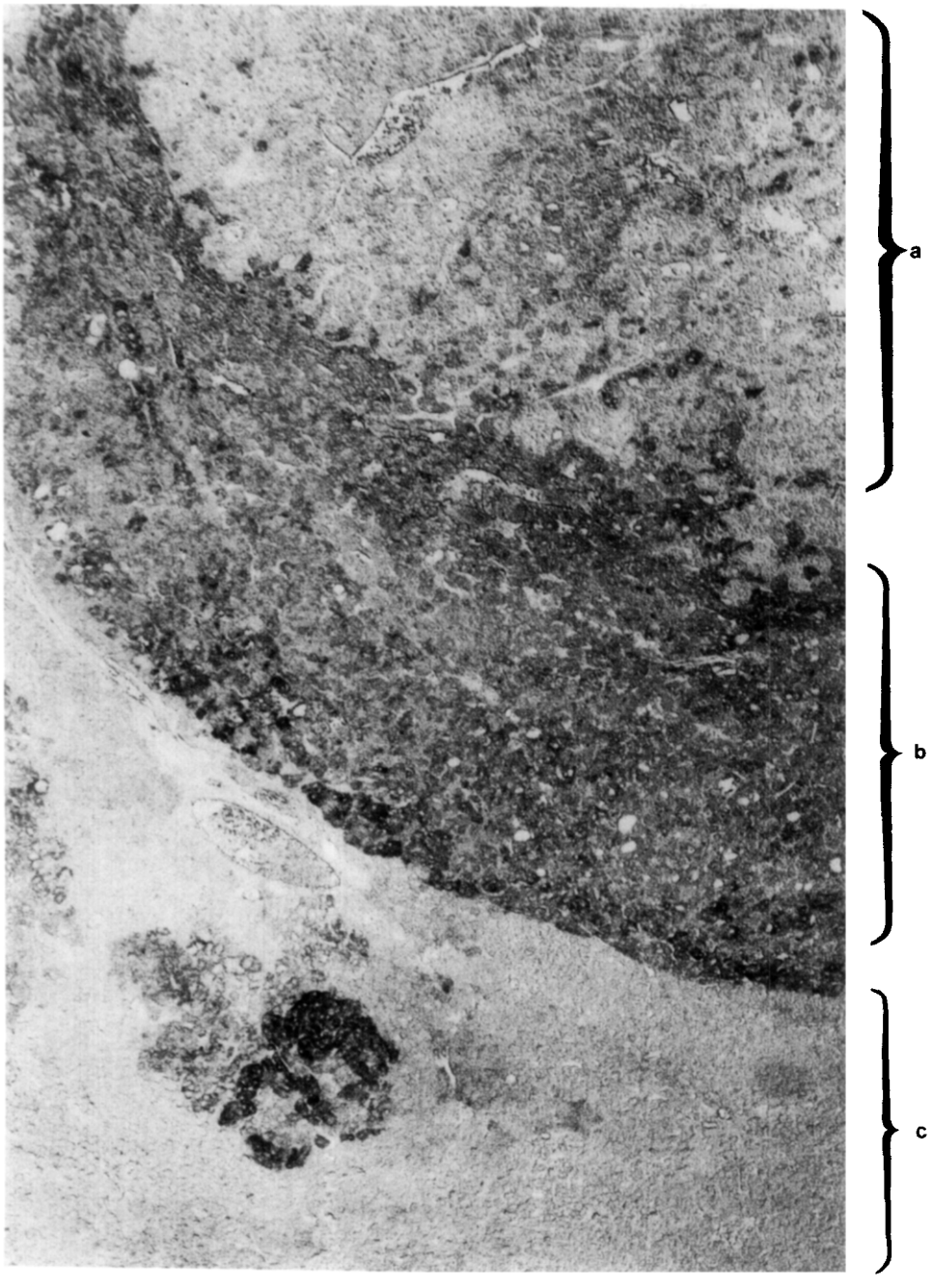

Fig. 3. Immunohistochemically (EH positive) stained section obtained from the liver of a rat 250 days after beginning of treatment with 10 mg/kg NNM, containing adjacent carcinoma (a), benign hepatoma (b), and normal tissue (c). Enlargement 1:300.

Benign and malignant tumors. Benign hepatomas started to occur frequently about 160 days after beginning of treatment with NNM. The

elevated EH (Figure 2B) and decreased ATPase (Figure 2A) levels were seen throughout all benign hepatomas investigated. In contrast EH levels were no longer elevated but ATPase deficiency was observed in all malignant hepatocarcinomas investigated. These hepatocarcinomas were AFP-positive, PAS negative, basophilic and had characteristic malignant morphology. The adjacent areas seen in Figure 3 clearly demonstrate this histochemical difference between malignant and benign tumors.

Thus, a focal increase in EH was seen at very early stages in hepatocarcinogenesis, and in the following stages including benign hepatomas, but was not observable in the malignant tumors investigated so far. The general occurrence of this difference and its potential applicability as a possible marker for discriminating between malignant and benign liver tumors are being further investigated.

ACKNOWLEDGEMENTS: We thank Professor P. Bannasch, Heidelberg, for histological examination of the tumors and the Deutsche Forschungsgemeinschaft for financial support.

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