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FERRITIN IS INCREASED IN DIETHYLNITROSAMINE - ALTERED RAT HEPATOCYTES

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Summary : Ferritin was localized by immunoperoxidase in rat liver during the early stages of experimental carcinogenesis induced by diethylnitrosamine. Carcinogen - altered hepatocytes, identified by their reduction in membrane-bound ATPase activity, showed a highly elevated content in ferritin (or ferritin subunits), compared to normal hepatocytes. This could correspond to an accumulation of free subunits in the cytoplasm or to a "non-specific" increase in ferritin synthesis related to the carcinogenic process. Our results suggest that some cytoplasmic proteins other than enzymes can be modified during the early stages of carcinogenesis and that ferritin accumulation detected by immunolocalization can be used as a valuable marker to identify foci of cellular alterations.

Introduction

A variety of cellular lesions are produced in rat liver by chemical carcinogens before hepatocellular carcinomas develop. Altered foci and neoplastic nodules are two types of lesions that have been precisely described (1, 2) and several enzyme histochemical reactions have been used as valuable markers for the identification of carcinogen-altered cells (3, 4).

Since these cells may display considerable phenotypic heterogeneity in their enzymatic properties (5), a new marker which identifies all hepatocellular lesions has been developped. With this procedure, it has been shown that the lesions are resistant to iron accumulation when the liver is overloaded with iron by dietary administration (6, 7). On the other hand, it has been well demonstrated that iron incorporation into normal hepatocytes results in an increase in the synthesis of ferritin (8, 9) which is the major cellular iron-storage protein. These different observations suggest that ferritin synthesis could be modified during the early stages of hepatocarcinogenesis. In order to verify this hypothesis, we performed immunolocalization of ferritin in rat liver during the first stages of carcinogenesis induced by diethylnitrosamine.

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Abbreviations : DENA, diethylnitrosamine ; SDS, sodium dodecyl sulfate ;
PAP, peroxidase - antiperoxidase
HEPES, N-2 Hydroxyethylpiperazine -N'-2-ethanesulfonic acid

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Material and methods

Material

Young adult female Sprague-Dawley rats were used. After two-third partial hepatectomy, the rats received three doses of DENA (25 mg/kg) by stomach tube, 24h, 48h and 72h later. Fifteen rats were sacrified at 3 and 6 months after DENA treatment and 5 female rats of the same strain were used as controls.

Immunological methods.

Ferritin was prepared from normal rat liver using the method of Drysdale et al. (10). The purity of the preparation was checked by SDS-polyacrylamide gel electrophoresis. Specific antiserum was raised in rabbits and purified by affinity chromatography on ferritin-CNBr Sepharose (Pharmacia, Uppsala, Sweden) column. Purified antibodies and normal rabbit immunoglobulins were coupled to peroxidase according to the two step procedure described by Avrameas and Ternynck (11). Sheep anti-rabbit γ-globulin antiserum was obtained from Institut Pasteur, Paris and peroxidase-anti-peroxidase complexes from Miles Yeda Ltd, Rehovot, Israël.

Tissue fixation

Fixation of liver was performed as previously described (12) with the following modifications: before fixation, rat livers were washed by perfusion via the portal vein with Hepes buffer for 30 sec. then cut into fragments and immersed in 10 % paraformaldehyde buffered with 0.1 M phosphate for 6-8h at 4°C. The samples were washed several times in phosphate buffer and soaked in 10% glycerol in phosphate buffer before freezing in liquid nitrogen-cooled isopentane. Eight-micron thick serial sections were performed for histochemical and immunoperoxidase reactions.

Histochemical staining of ATPase activity

Bile canaliculi-membrane bound ATPase activity was revealed by the Wachstein and Meisel method (13) using sodium ATP as a substrate. Incubations were performed for 1h at 37°C. Controls were obtained with omission of Mg^{++} or ATP in the incubation medium or with 10 mM p-chloromercuric benzoate as an inhibitor.

Immunolocalization of ferritin

Liver cryostat sections were processed for immunoperoxidase staining by two different methods a) by the PAP method first using anti-ferritin antibodies then anti-rabbit γ-globulin and finally peroxidase anti-peroxidase complexes according to Stenberger et al. (14) ; b) by the direct technique using peroxidase-labelled rabbit anti-ferritin antibodies. Peroxidase activity was demonstrated by the technique of Graham and Karnovsky (15).

Control reactions included : a) incubation with ferritin-absorbed rabbit antiserum ; b) with peroxidase labelled normal rabbit immunoglobulins ; c) with normal rabbit serum then with anti-rabbit γ-globulin and finally with peroxidase-anti-peroxidase complexes.

Results

The early morphological alterations induced by diethylnitrosamine were similar to those previously described by others (4). Altered hepatocytes revealed by their deficiency in membrane-bound ATPase activity formed small foci after 3 months. At 6 months, the lesions persisted and appeared as growing nodules.

Immunolocalization of ferritin by the PAP method and the direct technique yielded the same results: both in normal and in DENA-treated rat livers, all hepatocytes contained ferritin and no staining was observed in Kupffer cells.

However, in the carcinogen-altered hepatocytes, ferritin content was consistently higher than in the surrounding normal hepatocytes. As shown on serial sections, all ATPase-deficient hepatocytes located either in foci (Fig. 1) or in nodules (Fig. 2) displayed a strong staining for ferritin.

Discussion

Ferritin and its subunits have already been localized in rat liver (16) and in cultured rat hepatoma cells (17) by immunofluorescence: they showed a diffuse cytoplasmic fluorescence together with fluorescent "granules" in some cells. A very similar pattern was observed in our conditions with the immunoperoxidase technique. The absence of staining in the Kupffer cells suggests that there is little, if any, ferritin in these cells. This is in agreement with biochemical data (18) showing that most of the iron is deposited as hemosiderin.

The enhanced staining of foci and neoplastic nodules in DENA-treated rat liver was unexpected. As ferritin is synthesised in reponse to iron incorporation into the cells, the inability of chemically-altered hepatocytes to store iron should have resulted in a decrease in ferritin content. On the contrary an early and significant increase in intracellular ferritin content is observed. Several explanations could be proposed for these apparently contradictory results. Firstly a reduced rate in ferritin turnover or an increase in the endocytotic process could occur. The latter seems excluded since at the ultrastructural level, no endocytotic vesicles were seen (results not shown). Secondly a modification in the control mechanism by which iron regulates ferritin synthesis could lead either to a defect in the assembly of ferritin subunits into the completed shell or to an increase in ferritin synthesis. Iron has been shown to induce the formation of apoferritin from free

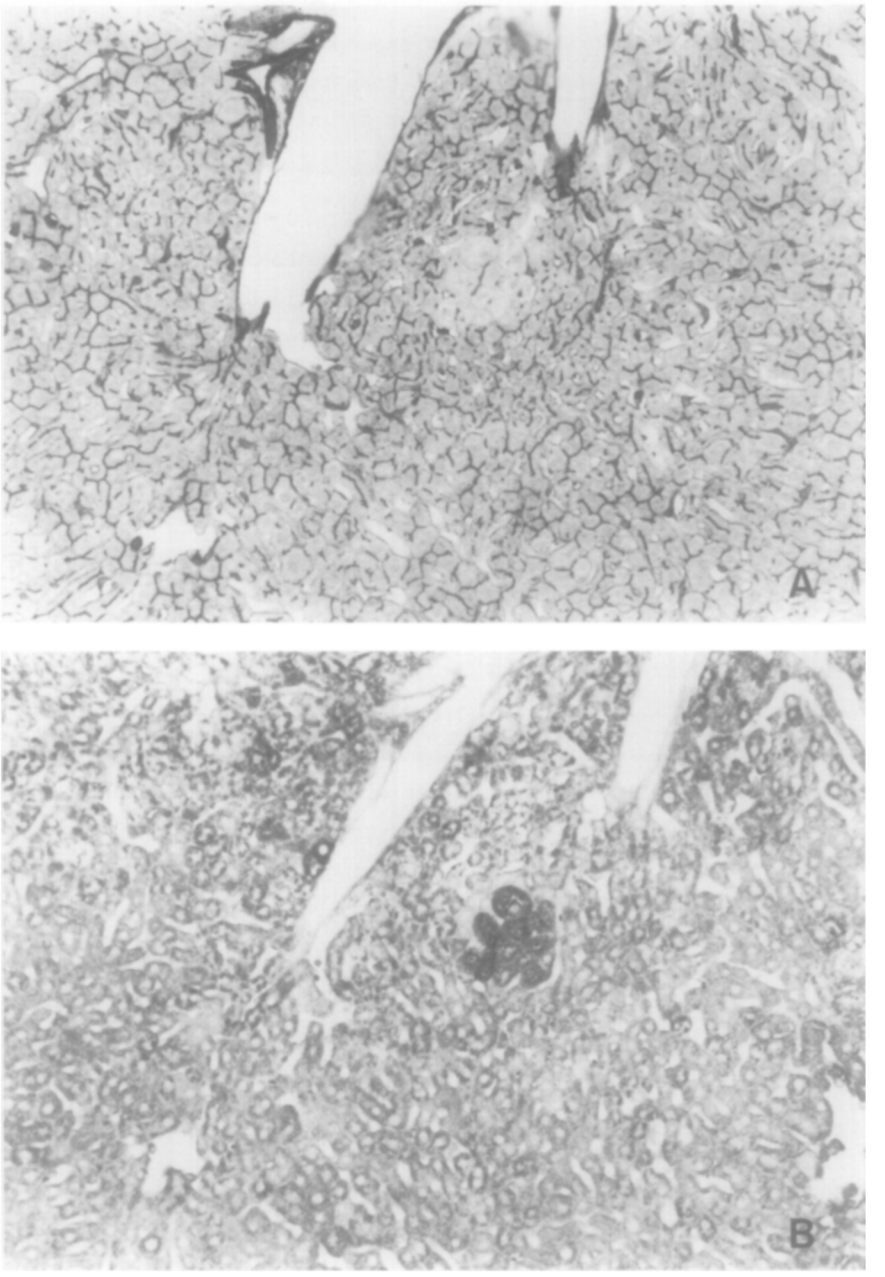

Figure 1 : Light microscopy ; serial cryostat sections of rat liver 3 months after DENA treatment ; A) ATPase reaction B) Immunolocalization of ferritin by the PAP method. The focus of altered hepatocytes exhibits a much stronger staining, as compared to surrounding normal hepatocytes (x 120).

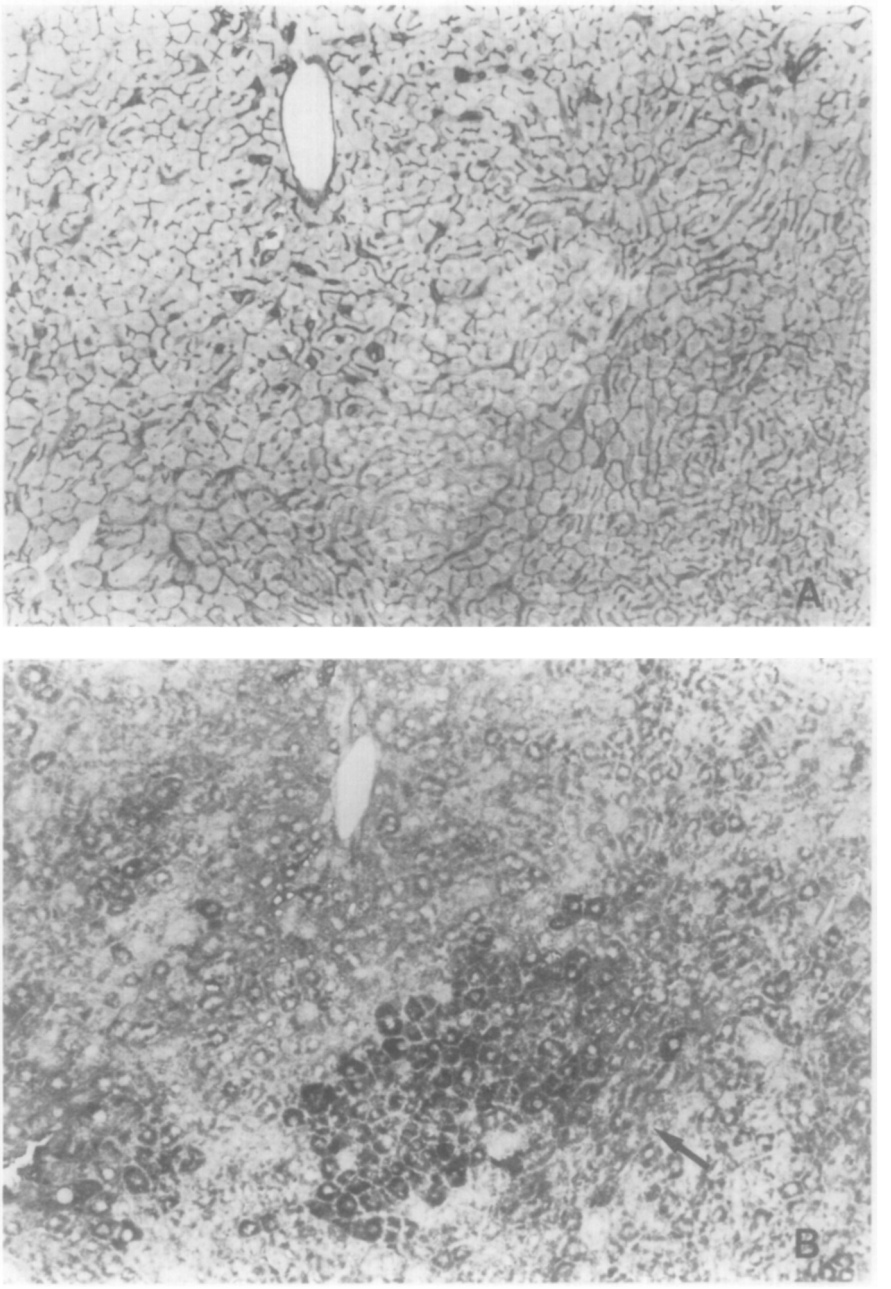

Figure 2 : Light microscopy ; serial cryostat sections of rat liver 6 months after DENA treatment ; A) ATPase reaction B) Immunolocalization of ferritin by the PAP method. The altered hepatocytes form a large island which appears as a developping nodule ; note the presence of some slightly compressed surrounding hepatocytes (arrow) (x 120).

subunits dispersed throughout the cytoplasm (19). Therefore the absence of iron in these altered cells could lead to accumulation of unengaged subunits. With the antibody used in this study, we cannot discern whether the observed staining corresponds to ferritin or to subunits or whether "non specific" increase in ferritin synthesis not stimulated by iron might occur. We suggest that this finding could be related to the high degree of ploidy which occurs in these hypertrophic altered cells (20). In this regard we have preliminary evidence that albumin synthesis increases with ploidy by studying normal rat hepatocytes separated by counterflow centrifugation in different subpopulations according to cell size. Whatever is the exact significance of this high ferritin content of carcinogen-altered hepatocytes, our findings are the first to our knowledge, to indicate that in these cells an intracytoplasmic protein other than an enzyme is modified. Moreover all foci and nodules identified by their deficiency in ATPase activity displayed a higher ferritin content. Consequently ferritin accumulation could be used as a reliable marker to detect early lesions occuring during experimental hepatocarcinogenesis.

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