Histochemical Studies on Nuclease Activity and Neoplastic Transformation in Rat Liver during Diethylnitrosamine Carcinogenesis

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SUMMARY

The distribution of RNase activity and the occurrence of hyperbasophilic foci and tumors have been examined in rat liver during diethylnitrosamine carcinogenesis.

A focal loss of RNase activity was found to occur in the hyperplastic liver nodules prior to the development of hyperbasophilic foci and hepatomas. The RNase-negative areas were widely distributed in livers of rats treated with diethylnitrosamine and so were the hyperbasophilic foci and the tumors. The results indicate that temporal and topographic correlations exist among these phenomena and suggest that a deficiency of RNase activity may play a role in the development of hyperbasophilic foci and hepatomas.

INTRODUCTION

Hyperplastic lesions induced by hepatocarcinogens are apparently essential to the development of hepatomas (7, 9, 11), and groups of hyperplastic cells characterized by an intense staining of cytoplasmic RNA with basic dyes seem to represent the actual sites of tumor origin (4). The mechanisms involved in the development of hyperbasophilic and neoplastic cells are still unknown. Recent studies have suggested, however, that a loss of RNase activity might play a role in the process since hyperplastic nodules of animals fed 4-dimethylaminoazobenzene or 3'-methyl-4-dimethylaminoazobenzene showed a focal loss of RNase activity prior to the formation of hyperbasophilic foci and hepatomas, which are also devoid of RNase activity (3).

The aim of the present study was to determine whether correlations among deficiencies of RNase activity, formation of hyperbasophilic foci, and tumor development could be observed with other hepatocarcinogens. Diethylnitrosamine, with a chemical structure completely different from that of the aminoazo dyes, is a highly potent carcinogen known to induce a widespread development of hepatocellular carcinomas in the rat (8, 10, 13). If RNase deficiency and formation of hyperbasophilic foci are significant changes in preneoplasia, they should show the same sequence in this system as with aminoazo dyes. In addition, RNase-negative areas and hyperbasophilic foci should be more widely distributed in rats receiving diethylnitrosamine. Such observations would indicate that these changes are consistently related and perhaps necessary for the neoplastic process.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 250 to 300 g were fed with Purina laboratory chow, and diethylnitrosamine was given in drinking water ad libitum at a concentration of 0.01%, during a 16-week period. Groups of 4 animals were killed at 2-week intervals by aortic exsanguination under ether anesthesia.

Preparation of Fixed Tissue Sections. Liver samples were fixed in Carnoy’s fluid for 24 hr, dehydrated through graded alcohols, and embedded in paraffin. Histological sections were cut at 5 μm, stained with toluidine blue, and mounted in balsam under a coverslip.

Histochemical Demonstration of RNase Activity. Fresh pieces of liver were placed on microtome stages, frozen at −10°, and sectioned at 10 to 15 μm in a cryostat. Sections were deposited on gelatin-RNA films and were incubated at room temperature in a moist chamber for periods varying from 15 min to 2 hr. Then the sections were removed by flushing with distilled water and the substrate films were stained directly with toluidine blue.

RESULTS

Degenerative and Regenerative Changes. Animals receiving diethylnitrosamine showed evidence of early degenerative changes in hepatic tissue. The formation of clear irregular spaces in the cytoplasm and occasional nuclear pyknosis were observed in periportal hepatocytes after a few weeks, while cytoplasmic hypobasophilia was noted in cen-
trolobular parenchyma. These changes were followed by necrosis of hepatic tissue in both sites.

The regenerative process started after 4 weeks. Mitoses were seen mainly in the intermediate zone of the liver lobules and the proliferation of hepatocytes led to the formation of multiple nodules. Connective tissue and bile duct infiltration was minimal, and only thin trabeculae usually separated adjacent hyperplastic nodules.

Various portions of the hyperplastic parenchyma exhibited hyperbasophilic properties after 8 weeks of diethylnitrosamine treatment. The phenomenon involved parts of nodules or whole nodules, and the hyperbasophilic areas occasionally extended over several adjacent nodules. A particular feature of the hyperbasophilic foci in these animals was their wide distribution (Fig. 1). Some hyperbasophilic areas showed no other morphological alteration while many exhibited additional cytological changes. In all cases however, the hyperbasophilic areas could be recognized as modified portions of regenerative nodules and did not represent outgrowths of smaller foci since no distortion of tissue architecture was associated with the presence of such areas in parenchymal nodules (Fig. 2).

Hepatomas were observed after 10 weeks of diethylnitrosamine treatment in the present experiment and were often widely distributed thereafter. The tumors were hyperbasophilic; they showed several cytological changes and alterations of tissue architecture (Fig. 3), as described for hepatomas induced by other carcinogens. Necrosis was common and was especially prominent in large tumor masses. Cholangiomas were rare and were found only in a few rats that had received diethylnitrosamine for 16 weeks.

**RNase Activity.** RNA films exposed to sections of normal rat liver revealed the presence of appreciable RNase activity in hepatocytes, in agreement with previous results (3). Fig. 4 shows a fresh-frozen liver section fixed in Carnoy's fluid and stained with toluidine blue; *arrowheads* indicate portal spaces. Fig. 5 illustrates a reaction obtained with an RNA film exposed to an adjacent serial section and stained with toluidine blue after removal of the tissue section. Areas of RNA hydrolysis appear light and indicate positive reactions; undigested areas appear dark and correspond to sites of low RNase activity. A comparison of these figures indicates that the hepatocytes of the periporal areas give a stronger reaction than those of the centrolobular areas in normal liver.

The distribution of RNase activity in hepatic tissue was not significantly altered after 2 weeks of diethylnitrosamine treatment, but negative areas were occasionally observed after 4 weeks. The RNase-negative areas increased in number and size at the following time intervals (Figs. 6 and 7) and often involved more than one-half of the liver parenchyma after a 10-week period. No other particular feature characterized the areas of hyperplastic nodules devoid of RNase activity.

The hyperbasophilic foci showed no appreciable RNase activity (Figs. 8 and 9). The hepatomas were consistently negative (Figs. 10 and 11). Hence, the livers at later stages of the carcinogenic process included RNase-negative areas corresponding to either hyperplastic parenchyma, hyperbasophilic foci, or hepatomas. The RNase activity shown by some hyperbasophilic foci and tumors after 16 weeks was observed to be associated with necrotic areas.

**DISCUSSION**

The sequence of changes induced by diethylnitrosamine is essentially the same as that noted with aminoazo dyes (6), *i.e.*, necrosis, hyperplasia, hyperbasophilia, and tumor development.

Hyperplastic nodules were found to give rise to hyperbasophilic foci after 8 weeks in animals receiving 0.01% diethylnitrosamine. Hepatomas were observed from 10 weeks in the present work but previous studies (4) have indicated that they may develop after an 8-week treatment in some animals. Both phenomena are apparently delayed with lower doses of diethylnitrosamine (8, 12). With aminoazo dyes, the induction period of hyperbasophilic foci and tumors is 8 weeks with the highly potent 3'-methyl-4-dimethylaminoazobenzene and 12 weeks with the less active 4-dimethylaminoazobenzene (4). The formation of hyperbasophilic foci and the development of hepatomas thus seem to be closely related in time in various carcinogenic conditions. Moreover, the fact that the hyperbasophilic foci showed a wider distribution in livers of rats fed diethylnitrosamine than in those of aminoazo dye-fed animals, as did the tumors, suggests that the 2 phenomena are also topographically related.

Histochemical analyses of RNase activity have revealed that diethylnitrosamine, as well as aminoazo dyes, induces a focal loss of RNase activity in hyperplastic liver nodules prior to the development of hyperbasophilic foci and hepatomas. Such an alteration was not observed in normal liver regeneration following partial hepatectomy (1). The change occurred after 4 weeks with diethylnitrosamine, as with 3'-methyl-4-dimethylaminoazobenzene which is of comparable carcinogenicity, while 8 weeks were required with the less potent 4-dimethylaminoazobenzene (3). Furthermore, of special interest is the fact that the ribonuclease deficiency resulting from diethylnitrosamine treatment involves a much larger proportion of hyperplastic liver parenchyma than the loss of RNase activity induced by aminoazo dyes. These results indicate that not only do the RNase deficiency, the formation of hyperbasophilic foci, and the development of hepatomas show a correlation in time, but they also show a correlation in topographical distribution. The present work thus strengthens the view that a loss of RNase activity may represent an alteration of hyperplastic hepatocytes that allows them to undergo other changes leading to a true neoplastic state.

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Figs. 1 to 3. Livers sections from rats fed diethylnitrosamine fixed in Carnoy’s fluid and stained with toluidine blue.
Fig. 1. Nodules of various sizes circumscribed by thin trabeculae of bile ducts and connective tissue in a rat treated with diethylnitrosamine for 12 weeks. Some nodules are totally hyperbasophilic and others only in part. × 15.
Fig. 2. Typical hyperbasophilic focus in a hyperplastic nodule after 8 weeks of diethylnitrosamine treatment. Cytological alterations were observed in the hyperbasophilic cells but no modification of the tissue architecture was noted. × 100.
Fig. 3. Hepatoma developing amid a hyperbasophilic area and compressing the adjacent cell cords in a rat treated with diethylnitrosamine for 16 weeks. × 100.
Figs. 4 to 11. Fresh-frozen liver sections from normal and diethylnitrosamine-treated rats fixed in Carnoy’s fluid and stained with toluidine blue (Figs. 4, 6, 8, and 10) and gelatin-RNA films exposed to adjacent serial sections and stained with toluidine blue after removal of the tissue sections (Figs. 5, 7, 9, and 11). The unstained areas of the films (light pattern resulting from the hydrolysis of RNA) correspond to the sites of RNase activity in the tissue sections. × 40.
Fig. 4. Liver section from normal rat. Arrowheads, portal spaces.
Fig. 5. RNA film exposed to adjacent serial section for 1 hr. Appreciable RNase activity is shown by the hepatocytes of the periportal areas, and a weaker reaction is given by the centrolobular hepatocytes. Arrowheads, portal spaces.
Fig. 6. Liver section from a rat treated with diethylnitrosamine for 10 weeks. Regenerative changes are the dominant feature at this stage.
Fig. 7. RNA film exposed to adjacent serial section for 30 min. Some areas of the hyperplastic nodules show relatively weak RNase activity.
Fig. 8. Liver section from rat treated with diethylnitrosamine for 16 weeks showing a hyperbasophilic focus on the left.
Fig. 9. RNA film exposed to adjacent serial section for 30 min. The hyperbasophilic hepatocytes are negative as well as the hyperplastic nodule on the right. Positive reactions are given by portions of other nodules.
Fig. 10. Liver section from rat fed diethylnitrosamine for 16 weeks showing part of a hepatoma on the left.
Fig. 11. RNA film exposed to adjacent serial section for 30 min. The tumor cells are RNase negative (left) but reactions are given by necrotic areas and the connective tissue stroma. The surrounding tissue gives reactions of varying intensities (right).
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