Effects of cell proliferation and cell death (apoptosis and necrosis) on the early stages of rat hepatocarcinogenesis

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An experiment was performed to investigate whether, during regression of the liver hyperplasia induced by a direct mitogen, apoptosis differentially affects replicated and non-replicated hepatocytes. After a single dose of the direct mitogen lead nitrate (LN), male Wistar rats were given repeated injections of tritiated thymidine, and were killed either 3 days (time of maximal hepatic DNA increase) or 15 days (complete regression of the hyperplasia) after mitogen treatment. Determination of liver DNA radioactivities and labelling indices (LIs) at the two time points revealed an ~40% loss in total liver DNA radioactivity, a 20% decrease in the specific activity of DNA, and a 20% reduction in the cell LI. Three days after LN administration 64% of the apoptotic bodies contained thymidine grains in their nuclear fragments. The results indicated that apoptosis affects both hepatocytes that replicated, and those that did not replicate, the former being slightly more sensitive. A second experiment was then performed to investigate whether and to what extent different types of cell death (apoptosis versus necrosis) influence the growth of hepatocytes initiated by a chemical carcinogen. Male Wistar rats were given a single dose of diethylnitrosamine, and 2 weeks thereafter either a single dose of LN, or a necrogenic dose of carbon tetrachloride (CCL₄). Bromodeoxyuridine was next infused for 5 days, and some of the animals were killed at this time point, and others after an additional 3 weeks. Administration of CCL₄ resulted in an increase in both the average size and the percentage area occupied by placental glutathione S-transferase-positive lesions. In contrast, administration of lead nitrate resulted in a strong reduction (50%) in the number of lesions with no remarkable change in the percentage area occupied by them. These differential effects occurred even though comparable LIs were observed in rats treated with the two agents. The results suggest that lead nitrate leads to a loss of initiated hepatocytes, due to the apoptosis that occurs during regression of the LN-induced hyperplasia.

Materials and methods

Animals and treatments

Male Wistar rats (Charles River, Milano, Italy and Japan SLC Inc., Hamamatsu, Japan) weighing 200–220 g were used. Diethylnitrosamine (DENA*; Sigma Chemical Co., St Louis, MO) dissolved in saline was given i.p. at a dose of 200 mg/kg body wt. Lead nitrate (LN; Carlo Erba, Milano, Italy) was dissolved in distilled water and injected through the femoral vein at a dose of 100 µmol/kg body wt. Carbon tetrachloride (CCL₄, Sigma) was dissolved in corn oil and given by stomach tube at a dose of 2 ml/kg. The animals had free access to food and water throughout the experimental period. After the animals had been killed, liver slices were fixed as indicated below, while the remaining liver was frozen in liquid nitrogen, and stored at ~80°C until further processing.

Experimental design

Experiment 1. Tritiated thymidine (NEN, Boston, MA; sp. act. 25 Ci/mmol) was injected i.p. at a dose of 10 µCi/100 g at 28, 32, 36, 40, 44, 50, 56, 62 and 70 h after treatment with LN or distilled water. Subgroups of rats were killed 72 h and 2 weeks after LN, or the vehicle.

Experiment 2. Two weeks after initiation with DENA, liver cell proliferation was induced by administration of either a single dose of LN (direct hyperplasia), or a necrogenic dose of CCL₄ (compensatory regeneration). Twenty-four hours thereafter, 30 mg/kg of BrdU (Sigma; 15 mg/ml in saline) was injected i.p., and immediately thereafter Alzet minipumps (Model 2ML1, Alzet Co., Palo Alto, CA), loaded with BrdU (33 mg/ml in 0.5 N NaHCO₃, rate of release 5 µl/h) continuously, were implanted s.c. into the backs of the rats. The minipumps were removed 5 days later and subgroups of rats were killed at this time point or 21 days later.

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Histology

In experiment 1, liver slices were fixed in 10% formalin, embedded in paraffin, and 5–6 μm thick sections were stained with H&E, or processed for autoradiography. Briefly, deparaffinized sections were dipped in NTB-2 nuclear emulsion (Eastman Kodak Co., Rochester, NY), exposed for 3 weeks, developed in D-19 Kodak developer, and counterstained with H&E. At least 1000 hepatocyte nuclei per section were scanned, and labelling indices (LI) were calculated as the fraction of labelled hepatocyte nuclei.

In experiment 2, liver slices were fixed in 95% ethanol containing 1% acetic acid, and paraffin sections were used for H&E staining, detection of BrdU-positive nuclei, and for scoring placental glutathione S-transferase (GSTP)-positive mini-foci.

Scoring of thymidine-labelled apoptotic bodies

Only apoptotic bodies (ABs) containing nuclear material were considered and quantitated as previously described (5). Those exhibiting thymidine grains in their nuclear fragments, following autoradiographic analysis, were defined as labelled ABs.

Measurement of BrdU- and GSTP-positive hepatocytes in DENA-initiated rat liver

BrdU incorporation into nuclei was determined immunohistochemically with a avidin–biotin peroxidase complex (ABC) method using a mouse anti-BrdU monoclonal antibody (Becton Dickinson Immunocytochemistry Systems, San José, CA) and Vectastain Elite ABC kit (PK-6101, Vector Laboratories Inc., Burlingame, CA). Briefly, tissue sections were deparaffinized, exposed to 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase, treated with 2% HCl, and incubated with normal horse serum for 20 min at room temperature. The sections were then incubated for 2 h with an anti-BrdU monoclonal antibody, followed by biotinylated horse anti-mouse IgG and avidin-biotin peroxidase complex. The sites of peroxidase binding were detected with diaminobenzidine (10), and the sections were counterstained with haematoxylin. A segment of duodenum, an organ with a high rate of cell proliferation, was included for each rat to confirm systemic delivery of the DNA precursor. In the liver tissues, at least 3000 hepatocyte nuclei per rat were scored.

The location of GSTP in the liver was determined immunohistochemically using an anti-rat GSTP monoclonal antibody (Medical & Biological Laboratories Co., Ltd, Nagoya, Japan) and Vectastatin Elite ABC kit (PK-6101, Vector Laboratories). The method was essentially similar to that described above except that 2 N HCl treatment was omitted and that normal goat serum and biotinylated goat and rabbit IgG were used. GSTP-positive mini-foci consisting of 10 hepatocytes were measured with a computer-assisted image analysis system comprising a Photomicroscope Vanox S (Olympus Optical Corp., Tokyo), a Color Image Processor SPICCIA II (Olympus), and a PC9801/VX personal computer (NEC Corporation, Tokyo) programmed for the three-dimensional calculation procedure of Campbell et al. (11).

Determination of total hepatic DNA content, DNA specific activity and total liver radioactivity

Tissue samples were homogenised in 6 vol of 0.075 M NaCl/0.025 M EDTA, pH 7.6, and were then precipitated in ice-cold 1 N perchloric acid (PCA). The pellet was washed several times until no radioactivity was detected in the supernatant fraction, and was then treated with 0.5 N PCA at 70°C for 1 h. Suitable aliquots of the hydrolisate were used for measurement of radioactivity in a Beckman LS 1801 liquid scintillation spectrophotometer, using Biofluor (NEN) as solvent. Radioactivity in a Beckman LS 1801 liquid scintillation spectrophotometer, using Biofluor (NEN) as solvent.

Hepatic DNA was quantitated by the Burton's diphenylamine method (12).

Statistics

Comparison between treated and control groups was performed by Student’s t-test.

Results

Experiment 1

In agreement with previous findings (5), a single injection of LN resulted in a 60–90% increase in liver weight and hepatic DNA content within 3 days (Table I). The increases were associated with a strong cell proliferative response, as shown by a 14-fold increase in total liver DNA radioactivity, and the DNA specific activity (Table I). Autoradiographic analysis revealed that both hepatocytes and non-parenchymal cells participated in the proliferative response. A striking increase in the labelling index of hepatocytes was present (see Table I), and even though the labelling was diffuse throughout the liver acinus, its intensity was greater in the perportal and midzonal regions. A clear regression of the mass and DNA content of the liver was observed 15 days after treatment (Table I), the decrease in DNA presumably reflecting apoptosis. The degree of cell loss was clearly indicated by a significant reduction (40%) of liver total radioactivity. On the other hand, the specific activity of DNA showed a reduction of ~20%. As a loss of non-parenchymal labelled cells could have been responsible for the reduction of specific activity of DNA, the LI of hepatocytes was also determined. The results shown in Table I indicate that a partial loss of hepatocyte labelling was present at the end of the regression phase. As shown in Table II, a significant number of ABs (1.41%), both intracellular and extracellular, was found in the liver of rats killed 3 days after LN administration. Autoradiographic analysis of the ABs revealed that 64% of them contained thymidine grains. Phagocytic activity by hepatocytes was evidenced by the presence of labelled ABs within the cytoplasm of thymidine-positive bacinucleate hepatocytes (Figure 1). Less than 0.01% ABs were observed in the liver of control rats, or in liver sections from rats sacrificed 15 days after LN treatment.

Experiment 2

As shown in Table III, 2 weeks after DENA administration ~580 GSTP positive foci/cm² were present in the liver, with a percentage area occupied by them of 0.18%. The average size and percentage area were only slightly different in rats of the same group sacrificed 3 weeks later (5 weeks from initiation). However, treatment with LN resulted in an approximately 50% loss of GSTP positive foci. Moreover, the proliferative effect of LN did not result in any increase of the percentage area of GSTP-positive lesions, even though a slight but significant decrease in the average size was evident, when compared to the group killed 5 weeks after DENA, suggesting the presence of a population of GSTP-positive hepatocytes, resistant to apoptosis, which underwent cell replication. After a 5 day infusion of BrdU, the LI of hepatocytes in LN-treated

<table>
<thead>
<tr>
<th>Table I. Liver weight, hepatic DNA content, total thymidine incorporation, DNA specific activity and LI during hyperplasia and regression following LN treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treatment</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Control (3 days)</td>
</tr>
<tr>
<td>LN (3 days)</td>
</tr>
<tr>
<td>LN (15 days)</td>
</tr>
</tbody>
</table>

Rats were treated with a single dose of LN (100 µmol/kg).[^1]H][H]Thymidine (10 µCi/100 g) was given at 28, 32, 36, 40, 45, 50, 56, 62, and 70 h after treatment. Rats were killed at 72 h and 15 days after LN or the vehicle.

a Mean ± SD of four rats per group of one representative experiment. Similar results were obtained in four different experiments.

b Significantly different from control for at least P < 0.005.

c Significantly different from LN (3 days) for at least P < 0.010.

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Table II. Incidence of labelled and unlabelled ABs 3 days after LN treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AB/100 nuclei</th>
<th>3H-labelled ABs</th>
<th>Unlabelled ABs</th>
<th>Labelled/unlabelled %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&lt;0.01</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LN (3 days)</td>
<td>1.41</td>
<td>162</td>
<td>91</td>
<td>64</td>
</tr>
</tbody>
</table>

Rats were treated as described in Table 1 and were killed 3 days after LN treatment. A total of 253 ABs were scored in liver sections of four rats. Less than 0.01% of ABs were found in liver sections of controls or rats treated with LN and killed 15 days afterwards. Only ABs containing nuclear fragments were examined.

ND; not determined.

Rats was 60% versus 20% in rats administered DENA alone (Figure 2). Treatment with DENA did not modify the proliferative response induced by LN. Several BrdU-positive apoptotic hepatocytes were observed (see Figure 3). The possibility of determining whether the ABs were present in the mini-foci or in the normal parenchyma was precluded by the fact that, as a consequence of LN treatment, almost all liver cells were positive for GSTP at this time point (13,14).

In contrast to these results, administration of a necrogenic dose of CCl₄, which resulted in a LI of hepatocytes of ~85% (see Figure 2), exerted a clear enhancing effect on the growth of GSTP-positive foci, as shown by a striking increase in the size (2.5-fold) and the percentage area (5-fold) occupied by

Fig. 1. Autoradiographic liver section from a rat killed 3 days after LN administration. A labelled AB lying within the cytoplasm of a binucleate hepatocyte is shown. Both the nuclei are also labelled.

Table III. Effect of LN and CCl₄ on the induction of GSTP-positive foci in rat liver initiated by DENA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GSTP-positive foci</th>
<th></th>
<th></th>
<th></th>
<th>Size distribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No./cm²</td>
<td>Size (mm²)</td>
<td>No./cm³</td>
<td>% foci area</td>
<td>Size (mm²)</td>
</tr>
<tr>
<td>Saline</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DENA (2 weeks)</td>
<td>7.9 ± 1.5</td>
<td>0.023 ± 0.023</td>
<td>582 ± 124</td>
<td>0.18 ± 0.04</td>
<td>81.8</td>
</tr>
<tr>
<td>DENA (5 weeks)</td>
<td>8.6 ± 1.6</td>
<td>0.034 ± 0.041</td>
<td>545 ± 128</td>
<td>0.29 ± 0.09</td>
<td>10.3</td>
</tr>
<tr>
<td>DENA + CCl₄</td>
<td>17.8 ± 2.3b</td>
<td>0.084 ± 0.081b</td>
<td>667 ± 40</td>
<td>1.49 ± 0.26b</td>
<td>46.4</td>
</tr>
<tr>
<td>DENA + LN</td>
<td>5.3 ± 0.9a</td>
<td>0.047 ± 0.038b</td>
<td>261 ± 41b</td>
<td>0.25 ± 0.10b</td>
<td>67.2</td>
</tr>
<tr>
<td>Saline + CCl₄</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Saline + LN</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are mean ± SD of five or six rats per group of one representative experiment. Similar results were obtained in three different experiments.

*aSignificantly different from DENA (5 weeks); P < 0.05.

*bSignificantly different from DENA (5 weeks); P < 0.005.
No GSTP-positive foci were observed when CCl₄ or LN were given in the absence of DENA (Table III).

Discussion

In previous studies, direct hyperplasia, unlike compensatory regeneration, was found not to support the evolution of chemical carcinogen-initiated cells to putative preneoplastic lesions (6,7). It was therefore suggested that hepatocytes initiated during the replicative phase were subsequently eliminated by apoptosis, during regression of the mitogen-induced liver hyperplasia. In those studies, however, no attempt was made to characterize the cells undergoing apoptosis (whether replicated, unreplicated or both), a question for which there is as yet no conclusive answer. For example, while Bursch et al. (8) suggested a selective elimination of non-replicated hepatocytes, after withdrawal of the liver mitogen cyproterone acetate, Oates et al. observed a random elimination of both replicated and unreplicated acinar cells of rat pancreas, following withdrawal of a mitogenic diet (9). In agreement with the latter findings, the present study shows that apoptosis occurring during regression of the liver hyperplasia induced by a single dose of LN affects both replicated and non-replicated hepatocytes. This conclusion is based on the finding that during regression of the induced hyperplasia (days 3–15) an ~40% fall occurred in both the mass of liver DNA, and the total radioactivity in liver DNA (Table I). At the same time, though, the specific activity of liver DNA decreased by only ~20%. Since LN induces proliferation not only of hepatocytes, but also of non-parenchymal cells, the possibility existed that a specific loss of the latter cell population could entirely account for the decrease of the specific activity of DNA. However, this possibility was excluded by the finding that LI of the hepatocytes from rats killed 15 days after LN also showed an ~20% decrease. Had apoptosis affected mainly or only non-replicated hepatocytes, no decrease or actually an increase in both the DNA specific activity and the LI should have been observed. Moreover, only ~60% of the ABs were found to be labelled by [³H]thymidine (Table II). Whether these effects are mitogen specific, or apply generally, remains to be established. Inasmuch as initiated cells, by definition, are cells that have

Fig. 2. LN- and CCl₄-induced liver cell proliferation. Hepatocyte LI in rats treated with a single dose of LN (100 μmol/kg, i.v.) or CCl₄ (2 mg/kg, i.g.) 2 weeks after DENA (200 mg/kg, i.p.) or saline administration. Labelling was done with 33 mg/ml BrdU administered via an osmotic pump implanted 5 days before necropsy. *Significantly different from DENA for P < 0.001.

Fig. 3. Immunohistochemical detection of BrdU using a monoclonal anti-BrdU antibody. Hepatocytes and non-parenchymal cells active in the cell cycle were observed by BrdU immunohistochesmy. BrdU-positive ABs are present within the cytoplasm of a binucleate hepatocyte (arrow) or in the extracellular space (arrowhead) in a liver section from DENA-initiated rats killed 6 days after LN. Hematoxylin counterstain.
undergone at least one round of replication, these results support the possibility that apoptosis occurring during liver hyperplasia may lead to deletion of initiated cells.

Apoptosis, however, is known to have a role at stages other than initiation, particularly at the promotion stage. Indeed, with the exception of a recent report indicating that apoptosis does not play a major role in counterbalancing cell proliferation in putative preneoplastic foci (15), several studies support the notion that apoptosis is critically involved during the promotion stage of hepatocarcinogenesis, as shown by the fact that (i) preneoplastic hepatocytes are characterized not only by a selective growth advantage, but also by an increased cell death index when compared to their normal counterpart (4,16); (ii) the apoptotic index increases with time (17); and (iii) promoting agents appear to inhibit apoptosis in both normal as well as preneoplastic cells (18,19). Interestingly, preneoplastic hepatocytes that exhibit a high sensitivity to apoptosis are also more resistant to necrosis induced by hepatotoxins (20).

A further aim of the present study was to examine whether apoptosis and necrosis, associated with direct hyperplasia or compensatory regeneration, would affect differently putative initiated cells (GSTP-positive hepatocytes) present in the liver after a single DENA exposure (21-23). In agreement with studies by others (24-27), our present data suggest that initiated cells are sensitive to apoptosis. Indeed, an ~50% reduction in the number of GSTP-positive lesions was observed in the liver of rats treated with LN. Why only a fraction of GSTP-positive lesions is lost, following LN treatment, is not known. It is possible that the persisting GSTP-positive lesions represent a different subset of initiated cells that possess an inherent resistance to cell death.

Interestingly, the loss occurs despite the strong mitogenic stimulus elicited by LN. A decrease in both the number and size of γ-glutamyltranspeptidase (GGT) and GSTP-positive lesions following chronic exposure to other inducers of liver growth, the peroxisome proliferators, has been previously reported (28-30). It could be argued that the decrease in the number of the enzyme-positive lesions is simply the result of the inhibition of GGT and GSTP expression, and not an actual regression of the lesions as in the case of peroxisome proliferators (31). However, LN, unlike peroxisome proliferators, is a strong inducer of hepatic GSTP (13,14,32).

The results obtained in the present study also show that the regenerative response elicited by a necrogenic dose of CCl₄, unlike the mitogenic stimulus induced by LN, exerted a promoting effect on the growth of GSTP-positive cells, as measured by the size of the GSTP-positive lesions and the percentage area occupied by enzyme-positive foci. As both proliferative stimuli induced powerful mitogenic responses in the liver, it appears that their opposite effect on GSTP-positive hepatocytes depends on the different type of cell death they induce—apoptosis and necrosis. It is well known that preneoplastic lesions induced by several carcinogenic regimens (with the exception of peroxisome proliferators) exhibit a special phenotype that makes them resistant to hepatotoxins, including resistance to necrosis (33). Thus, it is possible that hepatocytes expressing GSTP are already more resistant than normal cells to the necrogenic effect of CCl₄. Replication of these cells during the regenerative phase will give rise to a focal expansion. In contrast, it appears that a large fraction of putative initiated hepatocytes dividing after LN treatment may not be resistant to another type of cell death, apoptosis, induced by a homeostatic mechanism aimed at eliminating an excess of cells. The findings of the present study, indicating a different response of carcinogen-initiated hepatocytes to different types of cell death, may help to comprehend the conflicting data that exist in the literature on the relationship between cell proliferation and liver tumour formation (34,35).

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