Apoptosis and Cell Proliferation Are Involved in the Initiation of Liver Carcinogenesis by a Subnecrogenic Dose of Diethylnitrosamine in Refed Rats

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ABSTRACT We investigated whether changes in apoptosis and cell proliferation induced by starvation and refeeding in rat liver may contribute to the initiation mechanism of liver cancer by 20 mg/kg of diethylnitrosamine (DENA). Rats were starved for 4 d, then refed and given 20 mg/kg of DENA after 1 d of refeeding. Rat livers were examined before and after DENA treatment to measure DNA loss and synthesis, the number of placental GST-positive cells and their turnover. Four days of starvation depressed cell replication, as indicated by the labeling index (LI), and induced apoptosis, as shown by the decay of total DNA radioactivity and apoptotic index (AI, TUNEL technique). After 1 d of refeeding, AI significantly decreased and LI remained low, indicating that a high percentage of S phase cells was not required for the DNA damage due to 20 mg/kg of DENA. DENA induced apoptosis and the AI after 20 mg/kg of DENA was 3% in reared rats vs. 1% in fully-fed rats 5 d after DENA (P ≤ 0.05). Putative-initiated P-GST-positive hepatocytes appeared after administration of 20 mg/kg in reared rats, and they showed a higher LI (6%) than the surrounding P-GST-negative cells 3 d after DENA (LI = 2%; P ≤ 0.01), while very few P-GST-positive cells were found in fully-fed rats. These data indicate that starvation-induced cell loss and the subsequent refeeding trigger cell proliferation that gives a selective advantage to the cells initiated by 20 mg/kg of DENA to grow in the livers of refed rats. J. Nutr. 130: 104–110, 2000.

KEY WORDS: • diethylnitrosamine • rat liver • apoptosis • cell proliferation • cancer

Cell proliferation is essential in the genesis of cancer, particularly when chemicals are implicated. Several carcinogens induce tumors in various tissues or organs, but not in the liver unless they are associated with a proliferative stimulus (Farber 1991). A single dose of either methylnitrosourea (MNU) or dimethylhydrazine (DMH) becomes carcinogenic for the liver when administered after partial hepatectomy (PH) (Cayama et al. 1978, Craddock 1973). Cell replication seems to be necessary for chemical induction of initiated hepatocytes for at least two events: interaction of the carcinogen with the main target macromolecule, DNA, and fixation of the lesions by a round of cell division, in order to increase the chances of altered nucleotide sequences in the newly-made DNA (Farber and Sarma 1987). Nevertheless, initiated hepatocytes also occur when PH follows the administration of MNU, suggesting that the replication of carcinogen-damaged DNA before repair is the critical mechanism by which cell proliferation stimulates the induction of chemical initiation (Cayama et al. 1978, Columbano et al. 1981). Interestingly, several liver carcinogens are necrogenic and induce compensatory liver cell proliferation without the need for an exogenous mitogenic stimulus such as PH (Farber and Sarma 1987). A single 200 mg/kg dose of diethylnitrosamine (DENA) induces both extensive ethylation of liver DNA and cell necrosis, resulting in the generation of initiated hepatocytes selectively by a promoting regimen, eventually leading to liver cancer (Solt and Farber 1976). A 10–50 mg/kg dose of DENA is not necrogenic and does not induce hepatocellular carcinoma (Columbano et al. 1981, Ying et al. 1981). However, the subnecrogenic (20 mg/kg) dose of DENA becomes an initiating agent following PH, indicating that cell loss can be induced by either surgery or necrosis, to trigger a compensatory proliferative response (Ying et al. 1980).

We recently reported that administration of 20 mg/kg of DENA to previously starved and refed rats led to hepaticocyte foci, nodules and liver cancer without inducing cell necrosis, while if the rats received the full 200 mg/kg dose, refeeding had no effect (Tessitore et al. 1996). This implies that refeeding can substitute for PH or liver necrosis in inducing initiated hepatocytes by a subnecrogenic (noninitiating) dose of DENA. Consistently, Grassl-Kraupp et al. (1994) found that complete starvation of female rats for 8 d eliminated 20–30% of liver cells. Modulations of cell proliferation and cell death contribute to the growth of liver tumors (Cayama et al. 1978,

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Farber and Sarma 1987, Farber and Rubin 1991, Grassl-Kraupp et al. 1994, James and Muskhelishvili 1994), and this might also be true for tumor genesis.

Based on these observations, in the present study we tried to gain further insight into the mechanisms by which starvation leads to the induction of liver cancer by administering a subnecrogenic dose of DENA to refeed rats. For this purpose we investigated whether 4 d of starvation followed by 1 d of refeeding induced changes in the rates of active cell death and cell proliferation of rat liver which may be essential for the genesis of initiated hepatocytes following a subnecrogenic dose of DENA. We found that starvation and the carcinogen induced cell death and, as a consequence, the subsequent feeding of a basal diet produced a burst of DNA synthesis of the hepatocytes initiated by 20 mg/kg of DENA.

MATERIALS AND METHODS

Chemicals

\(^3\)H-thymidine (0.74 TBq/mmol) was from New England Nuclear (Boston, MA). Piccolfluor 40 was from Packard Instruments International (Zurich, Switzerland); calf thymus DNA, diethylnitrosamine and bromodeoxyuridine (BrdU) were from Sigma Chemical Co. (St. Louis, MO); NTB-2 Kodak emulsion was from Kodak (Rochester, NY); the “In situ cell death detection kit, fluorescein” was from Boehringer, Mannheim (Germany); the monoclonal mouse antibody to BrdU was from Amersham (Milan, Italy); the avidin-biotinylated peroxidase-complex and anti-mouse IgG antibody was from Vector (Burlingame, CA) and alkaline phosphatase-conjugated streptavidin and anti-rabbit IgG were from Dako (Carpinteria, CA). The rabbit anti-rat liver 7-7, glutathione S-transferase (placental isoform of P-GST) was a gift from Dr. Paolo Pani. All other reagents were from Merck (Darmstadt, Germany).

Animals and treatment

Six-wk-old male Fischer-344 rats were obtained from Charles River (Compo, Italy). They were maintained under a 12-h light/12-h dark cycle, controlled temperature (21 ± 1°C) and humidity (70–80%) and divided into three main groups. One group of rats was maintained for 4 d under conditions of total deprivation of food and then refed with the standard basal diet (AIN-76; Piccioni, Brescia, Italy; American Institute of Nutrition 1977). After 1 d of refeeding, 20 of these rats were given a single 20 mg/kg body wt dose i.p. of DENA. Groups 2 and 3 consisted of fully-fed control rats that were also exposed to P-GST antibody rabbit anti-rat diluted 1:500 for 1 h at room temperature, washed in phosphate buffer saline (PBS) and reacted with goat anti-mouse IgG diluted 1:200. After washing, sections were incubated with biotine-avidin-peroxidase complex. Visualization was achieved by reaction with 5-bromo-4-chloro-3-indoxyl phosphate and nitro blue tetrazolium chloride (BCIP/NBT). The sites with P-GST antigen were blue. A minimum liver area of 2 cm² was screened for each sample, using a Universal II microscope with 5× objective lens. The number of P-GST positive cells/cm² and the percentage of BrdU-positive cells were determined by means of an interactive image analysis system (Videoplan Zeiss; Kotron, Munich, Germany).

Cell death: end labeling

Sections 5 μm thick were stained with an enzymatic in situ label of apoptosis-induced DNA strand breaks, known also as the TUNEL (TdT-mediated dUTP nick end labeling) technique. The terminal deoxynucleotidyl transferase enzyme labels free 3′-OH DNA termini with fluorescein-labeled nucleotides. Percentages of apoptosing cells were determined by quantitating the percentage of labeled nuclei by fluorescence microscope, scoring not less than 5,000 adjacent cells (apoptotic index, AI).

Biochemical analyses

The liver was removed and weighed immediately after death. Liver samples were homogenized (100 g/L in distilled water) with a Polytron 10-ST apparatus (Kinematika, Luzern, Switzerland). Liver DNA content was determined by the method of Burton (1956) with calf thymus DNA as the standard. For radioactivity containing 9.6

Histological analyses

For histological/histochemical examination, slices taken from different liver lobes were immediately fixed in 10% phosphate-buffered formalin solution (pH 7) for routine embedding in paraffin and staining with hematoxylin and eosin. The mitotic figures were counted, and the mitotic index was calculated scoring not less than 2,000 cells.

Cell proliferation and initiation

Autoradiography. To calculate the percentage of \(^3\)H-thymidine-positive cells ([\(^3\)H-thymidine labeling index (LI)] some liver samples were processed for autoradiography: sections were coated with NTB-2 Kodak emulsion, dried and sealed in a dark box at ~80°C for 2 wk. Slides were then developed and counterstained with hematoxylin and eosin.

Immunohistochemistry. Other slices were fixed in ice-cold acetone for double immunohistochemical staining for BrdU and the placental form of glutathione S-transferase (P-GST) as described by Li et al. (1993).

To calculate the percentage of BrdU-positive cells, after deparaffinizing and rehydrating, sections were immersed in 0.08 mol/L of hydrogen peroxide for 10 min to block endogenous peroxidase; they were then immersed in 4 mol/L of HCl for 10 min to partially denature the DNA. The first antibody, anti-BrdU (mouse-indiluted antibody), was applied for 1 h at room temperature, washed in phosphate buffer saline (PBS), and reacted with goat anti-mouse IgG diluted 1:200. After washing, sections were incubated with biotine-avidin-peroxidase complex. Visualization was achieved by reaction with 5-bromo-4-chloro-3-indoxyl phosphate and nitro blue tetrazolium chloride (BCIP/NBT). The sites with P-GST antigen were blue. A minimum liver area of 2 cm² was screened for each sample, using a Universal II microscope with 5× objective lens. The number of P-GST positive cells/cm² and the percentage of BrdU-positive cells were determined by means of an interactive image analysis system (Videoplan Zeiss; Kotron, Munich, Germany).

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m mol/L sodium-deoxycholate for 3 h. The final hydrolysate was adjusted to pH 5 with 1 mol/L of HCl and radioactivity counted in a Beckman LS 1801 spectrometer (Milan, Italy), using Picofluor 40 (Packard, Warrenville RD) as scintillation mixture. The mean cell loss rates were calculated as fractional rates of DNA degradation (K_d) by the following equation:

\[ K_d = \frac{\ln (\text{total DNA radioactivity})}{t} \]

as previously reported (Tessitore et al. 1987).

**Statistical analysis**

Data are expressed as means ± SD and the data were analyzed by two-way ANOVA, followed by the Bonferroni-corrected t-tests. Differences were considered significant if P < 0.05.

**RESULTS**

The rats lost about 30% of their body wt after starvation for 4 d; weight increase was then linear even after administration of 20 mg/kg of DENA. The body wt of fed rats was restored 7 d after DENA treatment (Fig. 1, upper panel). Fully-fed rats receiving 20 mg/kg of DENA increased body wt normally, while fed rats injected with 200 mg/kg of DENA stopped growing. The food intake of previously starved rats on the first day of refeeding did not differ from that before starvation and to that of the fully-fed rats at the same time (Fig. 1, middle panel). While fully-fed rats treated with 20 mg/kg of DENA did not significantly change food intake (P < 0.001 vs. controls -5 d), those receiving 200 mg/kg of DENA immediately reduced food intake dramatically for 2 d (P < 0.001), then gradually returned to normal intake within 3–4 d. On the contrary, the previously refed rats progressively increased food intake after administration of DENA, thereafter remaining steadily at a significantly higher intake than controls (-5 d) for at least 4 d (Fig. 1, middle panel). Treatment with 200 mg/kg of DENA caused about 25% decrease (P < 0.0001 vs. -5 d) in liver weight while 20 mg/kg induced no significant change (P < 0.001 vs. -5 d) (Fig. 1, lower panel). Starved rats lost liver weight then quickly recovered on the first day of refeeding, the subsequent treatment with 20 mg/kg, producing a significant loss of liver weight (P < 0.001 vs. 0 d) (Fig. 1, lower panel).

Complete food withdrawal caused not only liver hypotrophy but also loss in DNA content (Fig. 2, upper panel). Liver DNA total radioactivity decreased over 4-d starvation, indicating a mean cell loss of about 18%/d (Fig. 2, lower panel).

After a single dose of thymidine labeled a dramatic decrease in both 3H-thymidine incorporation into DNA and LI was found after a 4 d starvation (P < 0.001 vs. 0 d), followed by an increase during refeeding but, surprisingly, the proliferative levels remained in all cases below the values in fully-fed rats.

**FIGURE 1** Effect of starvation and refeeding on body weight, food intake and relative liver weight of rats before and after diethylnitrosamine (DENA) treatment. Values are means ± so, n = 5. Refed + DENA 20 mg/kg †P ≤ 0.05, ††P ≤ 0.005 vs. DENA 20 mg/kg at the same time; DENA 20 mg/kg *P ≤ 0.05, ††P ≤ 0.005 vs. DENA 200 mg/kg at the same time; DENA 200 mg/kg †P ≤ 0.05, ††P ≤ 0.005 vs. refeeding + DENA 20 mg/kg at the same time.

**FIGURE 2** Effect of starvation on DNA content and total DNA radioactivity in rat liver. To study cell loss 40 rats received 7.4 MBq/rat 3H-thymidine by minipump infusion for 1 wk, then after a wk 20 rats were starved and 20 rats were fed and asphyxiated at different times after diethylnitrosamine (DENA) administration. Values expressed as mean of 5 rats ± so; *P ≤ 0.001 vs. time 0 d of starvation; starved rats †P ≤ 0.05, ††P ≤ 0.005, †††P ≤ 0.0005 vs. fed rats.
mg/kg of DENA to refeed rats also induced a significant increase in BrdU LI of P-GST-negative cells, and that of P-GST-positive hepatocytes was significantly lesser than that of P-GST-negative (rats treated with 20 mg/kg of DENA) the LI of P-GST-negative hepatocytes on d 3, following DENA injection, the BrdU LI of P-GST-positive hepatocytes was higher than that of P-GST-negative hepatocytes on d 3, whereas the LI of the P-GST-negative cells was different from that of the negative controls.

The LI of hepatocytes after 200 mg/kg of DENA rose to about 1% to d 7 after 20 mg/kg of DENA, the difference being even more pronounced vs. the levels of refed rats (0 d). Consistently, the liver mitotic index increased 5 and 7 d after administration of 200 mg/kg of DENA vs. 0 d (Fig. 3, middle panel). Interestingly, the rats previously starved and refeed also had significantly greater LI than control levels (P < 0.05). The LI of hepatocytes after 200 mg/kg of DENA rose to about 1% to d 7 after 20 mg/kg of DENA, the difference being even more pronounced vs. the levels of refed rats (0 d). Consistently, the liver mitotic index increased 5 and 7 d after administration of 200 mg/kg of DENA vs. 0 d, and after 20 mg/kg of DENA in refeed but not in fully-fed rats (Fig. 3, lower panel).

The induction of P-GST-positive cells, putative-initiated hepatocytes, following administration of a single dose of DENA, is illustrated in Figure 4 (upper panel). Single P-GST-positive cells were already evident 1 d after injection of 200 mg/kg of DENA, and their number increased progressively until d 7, while very few P-GST-positive hepatocytes were found in the liver of rats treated with 20 mg/kg of DENA. The refed rats showed a pattern of P-GST-positive cells similar to that of rats treated with 200 mg/kg of DENA, though to a significantly lesser extent (P < 0.005). Cell division, as P-GST-negative (Fig. 4, lower panel) hepatocytes. Following DENA injection, the BrdU LI of P-GST-positive hepatocytes in the positive control (rats treated with 200 mg/kg of DENA) was higher than that of P-GST-negative hepatocytes on d 3, and remaining significantly higher until d 7, while in the negative controls (rats treated with 20 mg/kg of DENA) the BrdU LI of P-GST-negative cells, and that of P-GST-positive cells was always below 2%. Interestingly, administration of 20 mg/kg of DENA to refed rats also induced a significant increase of P-GST-positive hepatocytes in the S phase vs. 0 d (P < 0.01) although the LI did not reach the high peak observed in the positive controls on d 3, whereas the LI of the P-GST-negative cells was not different from that of the negative controls.

DISCUSSION

The main findings of this research are that exposure of rats to starvation depresses cell proliferation and induces cell loss in the liver, resulting in a proliferative stimulus during subsequent refeeding, a high percentage of S phase cells is not required for DNA damage sufficient to trigger initiation by DENA in refeed rats, and a subnecrogenic dose of DENA triggers the appearance of P-GST-positive cells in refeed rats and allows their proliferation.

The depression of cell division in the liver of rats exposed to prolonged starvation that we observed is consistent with previous reports in normal tissue (Goodgame et al. 1979) as well as in hepatic foci and their surroundings (Hikita et al. 1997). The liver of starved rats also underwent apoptosis, probably as a consequence of the withdrawal of growth factors (Batistatou and Greene 1991, Chiao et al. 1995, Lindenboim et al. 1995, Straus 1994, Wang et al. 1995) or the induction of growth inhibitory factors, such as transforming growth factor beta (TGFβ) (Oberhammer et al. 1992). Induction of apoptosis by starvation was also reported in the liver of female rats (Grassl-Kraupp et al. 1994) and in both focal lesions induced by DENA and phenobarbital and the surrounding liver, again in female rats, resulting in the loss of detectable foci (Busch et al. 1990, Hikita et al. 1997). Refeeding following prolonged food deprivation is known to be associated with enhanced liver cell proliferation (Goodgame et al. 1979, Grube et al. 1985, Hikita et al. 1997). Refeeding is the proliferative stimulus which could make the rats susceptible to liver cancer induced by 20 mg/kg of DENA.

The importance of cell proliferation in the induction of carcinogenesis was extensively characterized in rat liver (Cayama et al. 1978, Colombano et al. 1981, Craddock 1971 and 1973, Kaufmann et al. 1991, Pittet et al. 1978). Adult rat liver is resistant to carcinogenesis; however, it becomes highly susceptible when hepatocyte proliferation is induced by the carcinogen itself or by PH. Kaufmann et al. (1991) studied the susceptibility of proliferating hepatocytes to complete initia-

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| **Effect of starvation and refeeding on 3H-thymidine incorporation into DNA and labeling index in rat liver**

<table>
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<tr>
<th>Dose of 3H-thymidine</th>
<th>Days of refeeding</th>
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<th>Refed</th>
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1 Values are means ± SD, n = 5, *P < 0.0005, **P < 0.0005 ***P < 0.0005 vs. 0 refeeding; †P < 0.05, ††P < 0.001, †††P < 0.0005, ††††P < 0.0001 fully-fed vs. refed.
2 Rats were starved for 4 d and refed for 1 or 2 d or continuously fed (fully fed). To evaluate 3H-thymidine incorporation into DNA and labeling index in the liver, rats received a single dose of 3H-thymidine i.p. (18.5 MBq/kg) 1 h before being asphyxiated or four 5.55 MBq/kg body wt doses of 3H-thymidine, i.p. every 6 h during the day before being asphyxiated.
tion by an alchylating agent administrated at different times after partial surgical hepatectomy; they reported that S phase liver cells appeared to be the most-sensitive targets for the DNA damage induced by the carcinogen. However, our finding that the percentage of S cells in refed rats at the time of DENA administration was lower than that in fully-fed rats rules out the possibility that a higher number of S cells contributed to the induction of DNA damaged cells by a subnecrogenic dose of DENA under our experimental conditions. On the other hand, our data suggest that rare cycling hepatocytes are sufficient to develop DNA damage, eventually leading to liver cancer.

The placental isozyme of GST is a marker of liver cancer development, as is the single hepatocyte that expresses P-GST an initiated cell (Cameron 1989, Dragan et al. 1993, Moore et al. 1987).

As previously reported (Dragan et al. 1993), the small number of P-GST positive hepatocytes induced by 20 mg/kg of DENA did not develop into foci, nodules or, overtime, cancer. In contrast, a relevant number of P-GST positive cells appeared in the liver of refed rats following injection of the same dose of DENA, consistently with the development of cancer.

At the same time, a large number of hepatocytes underwent apoptosis after administration of the carcinogen (Daoust and Morais 1986). Active cell death can result either in the elimination of cells with pro-mutational lesions, which may be at risk for initiation, and of about 80% of the initiated cells, or

**FIGURE 4** Effect of starvation on appearance of placental form of glutathione S-transferase (P-GST) positive cells and % S phase cells in rat liver after diethylnitrosamine (DENA) treatment. P-GST+/cm² = P-GST positive cells/cm²; % bromodioxouridine (BrdU) + P-GST+ = BrdU positive and P-GST positive cells/total P-GST cells; %BrdU + P-GST− = BrdU positive and P-GST negative cells/total P-GST negative cells. To evaluate the percentage of BrdU positive cells, that are cells in S phase, rats were given a single dose of BrdU i.p. 1 h before being asphyxiated 0, 1, 3, 5, 7 d after DENA administration, the appearance of P-GST+ initiated cells was evaluated by immunoistochemical analysis; values expressed as means of five rats ± sd; *P ≤ 0.05; **P ≤ 0.005 vs. time 0 d; refeeding + DENA 20 mg/kg †P ≤ 0.05, ††P ≤ 0.005 vs. DENA 20 mg/kg at the same time; DENA 20 mg/kg *P ≤ 0.05, **P ≤ 0.005 vs. DENA 200 mg/kg at the same time; DENA 200 mg/kg ‡P ≤ 0.05, ‡‡P ≤ 0.005 vs. refeeding + DENA 20 mg/kg at the same time.
in the reduction of preneoplastic and neoplastic lesions (Schulte-Hermann et al. 1995). Thus, induction of apoptosis was proposed as a novel preventive and therapeutic strategy against cancer. On the other hand, in rat liver, the apoptotic and proliferative rates increased from foci to adenoma to carcinoma, providing a selective advantage for growth to cell subpopulations with high levels of malignancy (Schulte-Hermann et al. 1995). Consistently, Grasl-Kraupp et al. (1994) showed that food restriction triggered apoptosis, and subsequent refeeding induced cell proliferation in liver focal proliferative lesions.

Our findings support the idea that starvation induced cell death without triggering liver cell necrosis until 7 d after DENA administration. However, liver cell necrosis until 7 d after DENA administration may be avoided by refeeding, as shown by a previous study (Kruse et al. 1985). Thus, the results obtained in this work confirm the hypothesis that DENA-induced liver cell necrosis may mean rates of cell loss sufficient to be the subject of further studies.

The differential cell proliferation in initiated hepatocytes and non-initiated liver cell population in refed rats indicate that a low differential is sufficient to activate the selective proliferative advantage necessary for initiation.

The enhanced rates of cell proliferation of initiated hepatocytes induced by DENA in refed rats further support the concept that replication of carcinogen-damaged DNA is critical in the initiation of carcinogenesis. The susceptibility to initiation depends on the replication of damaged DNA in critical cellular target genes, regulating the cell cycle before the gene's repair, leading to activation of the protooncogene and inactivation of the oncopressor genes. Gene alterations presumably occur with high frequency in S phase cells, replicating the ethylated DNA (Farber and Sarma 1987; Kauffmann et al. 1991, Singer 1986), probably because protooncogenes and oncopressor genes are more prone to mutation during their replication (Li et al. 1993, Oztruk 1991). Increased formation of adducts to DNA may be expected in rats previously exposed to refeeding; cell division must also occur before the carcinogen-induced critical lesion(s) in DNA have been repaired (Kauffmann et al. 1991). Interestingly, DNA damage produced in rat liver by DENA appears to be rapidly repaired in noncycling hepatocytes (Tates and Den Engelse 1989). The enhanced cell proliferation of initiated hepatocytes in refed rats did not rule out a direct effect of food deprivation/refeeding on metabolism of DENA, which will be the subject of further studies.

These results appear of particular interest for the pathogenesis of the hepatocellular carcinoma in underdeveloped countries, where the tumor is endemic and where aflatoxin B1 and hepatitis B virus are the main factors involved, and a condition of food-deprivation/refeeding may be present. This may also be true in developed countries, for people with unbalanced dietary habits.

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LITERATURE CITED


