Reversible Inhibition of Rat Hepatocyte Proliferation by Hydrocortisone and Its Effect on Cell Cycle-dependent Hepatocarcinogenesis by N-Methyl-N-nitrosourea

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INTRODUCTION

Hepatocytes in adult rats generally are resistant to carcinogenesis by a single exposure to agents capable of inducing cancer in other tissues (6, 17, 18, 31). For example, a single dose of the direct-acting carcinogen MNU* may induce tumors in rat breast, gastrointestinal tract, lymphoreticular system, skin, kidney, odontogenic tissues, brain, lung, ovary, uterus, and several endocrine and exocrine glands, but in no instance in which MNU was given to rats with normal livers did hepatocellular tumors develop (17, 18, 21, 22, 30). Hepatocyte susceptibility to carcinogenesis can be increased by certain treatments which stimulate the proliferation of damaged cells (6–9, 25, 26, 31). It has been shown recently that the stimulus for hepatic cell division induced by a two-thirds partial hepatectomy is necessary for the initiation of hepatocarcinogenesis by MNU (4). Of additional interest was the observation that hepatocytes were much more sensitive to initiation when treated with MNU shortly after, rather than before, the partial hepatectomy. This result complemented another study which had shown that MNU was hepatocarcinogenic when given as a single dose 24 or 31 hr after partial hepatectomy but not when given at 6 hr (9). Experiments reported here were designed to investigate whether the increased sensitivity of proliferating hepatocytes to carcinogenesis by MNU is related to the specific susceptibility of a particular phase of the cell division cycle.

The adrenal corticosteroid, hydrocortisone, has been shown to inhibit hepatocyte proliferation in regenerating livers (27, 28). The inhibition is only temporary and is reversible, so that appropriately timed treatment with hydrocortisone can be used to prolong the interval between the partial hepatectomy and onset of DNA synthesis by hepatocytes (28). By using hydrocortisone to modify cell progression in regenerating rat liver, it was possible to assess sensitivity to hepatocarcinogenesis in terms of the absolute time of treatment with MNU after hepatectomy and in terms of biological time as specified by progression of hepatocytes through the cell cycle. A preliminary account of this work has appeared (16).

MATERIALS AND METHODS

Chemicals. Chemicals and solvents were reagent grade and were used without further purification. MNU was from Ash Stevens Inc., Detroit, Mich. Hydrocortisone hemisuccinate was a gift from The Upjohn Company, Kalamazoo, Mich. [3H]dThd (specific activity, 19 or 59 Ci/mmole) in sterile H2O was from Schwarz/Mann, Rockville, Md. NTB-2 nuclear track emulsion was from Eastman Kodak Co., Rochester, N. Y.

Animals, Partial Hepatectomies, and Postsurgical Hydrocortisone Treatment. Fisher 344 male rats weighing 80 to 120 g were obtained from Charles River Breeding Laboratories, Inc., North Wilmington, Mass. Rats were maintained on a 12-hr (6:00 a.m. to 6:00 p.m.) light schedule and fed Purina laboratory chow and water ad libitum. While under light ether anesthesia, rats were subjected to a two-thirds partial hepatectomy (14) performed by the same investigator in all studies. Virtually all animals survived surgery. Operations were performed between 10:00 a.m. and 1:00 p.m. Hydrocortisone hemisuccinate was prepared as a fine dispersion at 50 mg/ml in filter-sterilized 0.15 M NaCl immediately prior to use. At 9, 14, and 19 hr following partial hepatectomy, the suspension was injected I.P. at doses of 50 mg hydrocortisone per kg body weight.

Characterization of the Kinetics of Hepatic Cell Proliferation following Two-thirds Partial Hepatectomy. At selected times after the partial hepatectomy, 0.4 mCi [3H]dThd per kg body weight was administered to rats by I.P. injection. After 30 min, animals were anesthetized with ether and exsanguinated by cutting the portal vein and abdominal aorta. Liver remnants were removed and placed in ice-cold 0.3 M

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sucreose containing 5 mm Na3EDTA before being further processed. A portion of the right lateral lobe was homogenized in a loose-fitting Dounce homogenizer with 15 volumes of 0.3 m sucrose containing Na3EDTA. Aliquots of the homogenate were frozen at -20° until all livers in an experiment had been similarly treated. Thawed homogenates were processed by the procedure of Shibko et al. (29) for quantitation of the DNA specific activity (dpm/μg DNA). The remaining liver was fixed in 10% neutral formalin; paraffin-embedded sections of the right lateral lobe were deparaffinized, dipped in Kodak NTB-2 nuclear track emulsion, and stored in light-tight slide boxes with desiccant for 6 to 8 weeks at 4°. Autoradiograms were developed using standard photographic techniques and stained with Harris' hematoxylin. Under an oil immersion objective, autoradiograms were scanned through the widest part of the lobe. A celluloid disk containing the letter Z drawn with a fine line pen was inserted into a microscope ocular. The letter extended across 62% of the ocular field measured horizontally and vertically. Continuous fields were viewed, and the numbers of labeled and unlabeled nuclei observed in hepatocytes lying directly beneath the letter line were counted. This procedure allowed a random selection of hepatocytes for analysis. Binucleate cells were counted as one cell. The numbers of hepatocytes with mitotic nuclei (prophase, metaphase, anaphase, or telophase) were determined similarly. At least 500 nuclei were scored for each liver section, and the labeling index (fraction of hepatocytes in S phase) and mitotic index (fraction of hepatocytes in mitosis) were determined for each animal.

Carcinogen Treatment. MNU was dissolved immediately prior to use in filter-sterilized 20 mm citrate buffer, pH 5.0, containing 0.15 m NaCl. Animals were anesthetized with ether, the midline incision from the preceding partial hepatectomy was reopened and extended caudally, and the intestines were retracted to allow access to the portal vein. Using a 30-gauge needle, 10 ml MNU solution per kg body weight were injected into the portal vein. The needle was removed, and a piece of Gel-foam was placed over the needle puncture site. The intestines were replaced, the midline incision was sutured, and the skin was closed with stainless steel clips.

Partially hepatectomized rats received 0.4 mmol MNU per kg intraperitoneally at 6, 20, 24, 30, 36, or 44 hr after surgery. At least 20 animals were treated at each time point. In another experiment, hepatic cell proliferation following partial hepatectomy was temporarily blocked by administration of hydrocortisone (50 mg/kg) 9, 14, and 19 hr after surgery. Groups of about 20 hydrocortisone-treated animals received MNU intraperitoneally 6, 24, 28, 32, 36, or 44 hr after partial hepatectomy. Initially, a dose of 0.5 mmol MNU per kg was given. This dose was reduced to 0.4 mmol MNU per kg in a second experiment to reduce the excessive posttreatment mortality associated with exposure to the higher dose. Three groups, each consisting of more than 30 animals, served as controls. One group was subjected only to partial hepatectomy. Another was subjected to partial hepatectomy and 3 hydrocortisone injections as described above. The third group was subjected to laparotomy only.

Long-Term Animal Holding, Necropsy Procedure, and Histopathology. Animals were housed in polycarbonate cages as described above. Animals were checked daily for deaths or moribund condition. Survival-impairing abnormalities such as overgrown teeth or abscesses along the surgical incision scar were treated to maintain the animal on test. Rats were necropsied according to the following procedure when found dead, when killed in apparently moribund condition, or when killed in apparently healthy condition at the conclusion of the study, 75 weeks after partial hepatectomy. Liver, kidneys, adrenals, stomach, intestines, spleen, eyes, brain and spinal cord, testes, and thyroid were routinely fixed in 10% neutral formalin and processed for histology. All other tissues were examined for gross lesions; observable lesions were preserved and processed. All livers were trimmed by cutting serially at 2- to 3-mm intervals, and the cut surfaces were examined for lesions. All liver lesions were processed; when none were found, 2 representative sections were processed.

Tumor incidences in the experimental groups were compared in 2 x 2 tables using the $\chi^2$ test. Tumor yields were compared using Student’s t test.

RESULTS

Kinetics of Hepatic Cell Proliferation after Partial Hepatectomy. The mean values of the DNA specific activity (dpm/μg DNA), the hepatocyte labeling index, and the hepatocyte mitotic index were determined at various times after partial hepatectomy (Chart 1). DNA-synthetic activity in liver remnants remained at low levels for up to 11 hr postoperatively but increased rapidly thereafter. The maximum values of the DNA specific activity and hepatocyte labeling index were reached between 17 and 20 hr when about 30% of the hepatocytes were synthesizing DNA (S phase). This represents an increase of 40-fold over the values observed in intact livers or at 6 hr after partial hepatectomy. A second wave of DNA synthesis, as reflected by the DNA specific activity and hepatocyte labeling index, reached its maximum at 36 to 40 hr. Although the liver DNA specific activity was about the same when DNA was labeled at 17 or 36 hr, the hepatocyte labeling index was reduced about one-third in the second peak. The additional increment of DNA synthesis activity at 36 hr after partial hepatectomy paralleled the entry of proliferating littoral and ductal cells into the S phase, as observed by autoradiography (10), after the first wave of hepatocyte DNA synthesis was completed. The transition of replicating hepatocytes into mitosis was abrupt and dramatic. At 22 to 24 hr after hepatectomy, maximal fractions of hepatocytes (about 4%) were undergoing mitosis, representing an increase of about 40-fold over the fraction in mitosis at 17 and 20 hr. After the peak at 24 hr, the mitotic index declined to 50% of the peak value between 30 and 40 hr. A second peak of mitosis by hepatocytes occurred...
at 44 hr at which time 2.6% were in M. Hepatocyte labeling and mitotic indices described similar curves but with the mitotic index curve lagging behind the labeling index curve by about 8 hr. When corresponding time points at the peaks and nadirs were compared, the ratios between the labeling and mitotic indices were 7 to 8. This reflected the relative durations of the S and M phases, 7 to 8 hr and 1 hr, respectively (10, 11).

**Effect of Hydrocortisone on Hepatic Cell Proliferation.**

Hydrocortisone was used to inhibit hepatic cell proliferation after partial hepatectomy (27, 28) and, so, to modify the kinetics of hepatocyte proliferation in regenerating livers. Administration of 50 mg hydrocortisone per kg body weight 9, 14, and 19 hr after partial hepatectomy delayed the initial onsets and peaks of DNA synthesis and mitosis by hepatocytes (Chart 2). At 20 hr after partial hepatectomy, the labeling index in hydrocortisone-treated rats was reduced about 90%, i.e., to 10% of that seen in rats subjected to partial hepatectomy alone. At 24 hr, the hepatocyte mitotic index in hydrocortisone-treated rats was 5% of that in untreated regenerating liver. Entry of hepatocytes into the S phase accelerated as the hydrocortisone block decayed; a maximum fraction of 38% of hepatocytes was in S phase at 32 hr after partial hepatectomy or 12 to 15 hr later than when the first peak was seen in hepatocellularized rats not given hydrocortisone. A rise and decline in the hepatocyte mitotic index followed the rise and decline in the labeling index by about 8 hr. Peak rates of mitosis occurred between 36 and 40 hr after partial hepatectomy, again about 15 hr later than the first peak in hepatocellularized rats not given hydrocortisone.

**Toxic and Carcinogenic Effects of Intraportal MNU.** Preliminary acute toxicity studies indicated that a dose of 0.88 mmol MNU per kg administered by portal vein injection during the first wave of hepatic cell DNA synthesis was lethal to all rats treated. A dose of 0.66 mmol MNU per kg was lethal to 75% of the animals treated between 6 and 48 hr after partial hepatectomy, while 0.5 mmol MNU per kg killed 4 of 10 animals treated between 24 and 32 hr after partial hepatectomy. Based upon this result, the 0.5-mmol MNU per kg dose was chosen initially for these experiments. Although sufficient numbers of animals treated at this dose survived to be included among our experimental groups, for subsequent studies, a less toxic dose of 0.4 mmol MNU per kg body weight was substituted. Consequently, all groups receiving partial hepatectomies but no hydrocortisone and all additional rats which did receive injections of hydrocortisone were treated with this lower dose of MNU. Rats in all groups, at both MNU dosage levels and with or without hydrocortisone treatment, sustained nonlethal but permanent toxic damage to the retina and developed a wide variety of tumors in extrahepatic tissues.

In the majority of experimental animals, tumors were found at multiple sites, with as many as 10 primary extrahepatic tumors/animal. Principal sites for tumor induction by MNU included the central and peripheral nervous systems, gastrointestinal tract, kidney, thyroid, Zymbal's glands, peritesticular mesothelium, and odontogenic tissues (Table 1). The only significant (>95% confidence interval) difference between rats given hydrocortisone and those subjected only to partial hepatectomy was in tumors of the intestines. In rats given the hormone, a lower tumor incidence was observed when MNU was administered within 24 hr after partial hepatectomy, i.e., during the time that the hydrocortisone-induced blockade of hepatic cell proliferation was effective. This result suggests that the effects of hydrocortisone were not specifically limited to the liver.

Except for restructuring of lobular architecture caused by postsurgical regeneration of residual liver, in livers of treated animals surviving 25 weeks, hepatic histology was normal at all sites not occupied by tumors. Cirrhosis, necrosis, and reactive hyperplasia were not observed. Tumors were identified grossly as solitary nodules, generally 1 cm or more in diameter. All but one hepatic epithelial tumor, a cholangiocarcinoma, were hepatocellular in origin. The majority (78%) of hepatic tumors were diagnosed as hepatocellular carcinomas on the basis of the following criteria: compression of surrounding parenchyma with incompletely defined margins; thickening of
liver cell plates into trabeculae more than 2 cells in width (Fig. 1), with or without formation of pseudoacini (Fig. 2); and increased mitotic activity.

A small proportion of hepatocellular tumors exhibited expansive growth with compression of, and lack of continuity with, the plates of adjacent hepatic parenchyma but differed in that they lacked the trabecular morphological pattern typical of hepatocellular carcinomas (Fig. 3). The majority of cells in these relatively large (1-cm diameter) lesions were uniform in morphological characteristics and contained single nuclei with an extremely prominent central nucleolus. These features are characteristic of classic neoplastic nodules (15). The natural history of these better differentiated lesions has not been defined for rats given single exposures to alkylating agents. As they possess certain features of neoplasia, including progressive growth in the absence of continuing exposure to the inducing agent, we considered them to be true neoplasms and therefore have grouped them with hepatocellular carcinomas in some tabulations.

No metastases from primary hepatocellular tumors were observed. The latency for the appearance of hepatic tumors was variable; however, 77% were observed among rats surviving for more than 45 weeks. No tumors were observed in livers of control rats, all but one of which (101 of 102) survived to terminal sacrifice 75 weeks after treatment. A fact of importance is that hepatocellular tumors were never the cause of death. In virtually all animals with liver cell tumors, death was the consequence of a lethal tumor of extrahepatic origin. Therefore, all hepatocellular tumors were incidental findings and in this regard statistically equivalent for the purpose of comparing frequency of tumors in different groups. For the most part, there was rather comparable survival among groups in each of the 3 experiments (Tables 2 to 4). Animals not surviving 25 weeks were excluded from consideration.

Table 2 summarizes hepatocarcinogenesis in rats not given hydrocortisone but administered 0.4 mmol MNU per kg 6, 20, 24, 30, 36, or 44 hr after partial hepatectomy. Incidences and yields of both forms of hepatocellular neoplasia demonstrated the same trend, generally being largest in rats treated at 20 hr or at a time when maximum fractions of hepatocytes were synthesizing DNA. Hepatocarcinogenesis appeared to be a significantly less likely development in rats treated either before or after the first peak of DNA synthesis in regenerating livers.

When hydrocortisone was used to increase the interval after partial hepatectomy before successive onset of DNA synthesis and mitosis by hepatocytes, the times of peak susceptibility to hepatocarcinogenesis also were shifted (Tables 3 and 4). Although the yields and incidences of hepatocellular tumors were small in rats given 0.4 mmol MNU per kg (Table 3), the maximum incidence was seen in rats treated at 32 hr during the peak of DNA synthesis. In rats given 0.5 mmol MNU per kg (Table 4), hepatocarcinogenesis was maximal in the group treated at 28 hr after partial hepatectomy or when proliferating hepatocytes were late in G1. Of 7 rats treated at this time, 6 or 86% developed hepatocellular carcinoma.

**DISCUSSION**

This study describes the kinetics of hepatocyte proliferation in the regenerating livers of partially hepatectomized juvenile
The typical pattern of restorative hyperplasia occurred and the first peak. Of DNA synthesis in hepatocytes during only the first wave. Of liver regeneration. In rats given a partial hepatectomy but not treated with hydrocortisone, DNA synthesis as detected by incorporation of [3H]dThd into DNA was an accurate reflection of DNA synthesis in hepatocytes during only the first wave. During the second wave, the entry of mitotic and ductal cells into S phase accounted for a substantial share of the labeling found by [3H]dThd incorporation while the second peak in the hepatocyte labeling index curve was reduced in comparison with the first peak.

Hydrocortisone injections given at 9, 14, and 19 hr after partial hepatectomy reversibly inhibited cell proliferation in regenerating livers delaying the onsets of DNA synthesis and mitosis by about 15 hr. Instead of a biphasic response of DNA synthesis and mitosis by hepatocytes, only single peaks were demonstrated during the period of observation. Rizzo et al. (28) initially reported that a single injection of 50 mg hydrocortisone per kg given to 240- to 260-g Sprague-Dawley rats at 19 hr after partial hepatectomy delayed the onset of DNA synthesis by about 10 hr. We were unable to attain adequate inhibition of DNA synthesis in 80- to 120-g Fischer 344 rats by a single injection of 50 mg hydrocortisone per kg given at 14 or 19 hr after partial hepatectomy. Our 3-dose regimen began at 9 hr after hepatectomy or 2 to 5 hr before the initial onset of DNA synthesis by hepatocytes in these rats. In the previous study, the single injection was given to 2 hr before the onset of DNA synthesis. Thus, hydrocortisone appears to inhibit cell proliferation reversibly by blocking hepatocytes at a point in the latter third of G2. Some slippage of the block may account for the gradual increases in labeling and mitotic indices between 20 and 28 hr. Analysis of the kinetics of hepatocyte proliferation in regenerating livers indicated that similar proportions of hepatocytes passed through the S phase between 10 and 30 hr after hepatectomy in rats not given hydrocortisone and between 24 and 44 hr in rats given the compound. Our data therefore confirm the observation made previously (28) that the effects of hydrocortisone on the proliferation of hepatocytes are largely reversible.

The hepatocyte mitotic index in hydrocortisone-treated livers peaked at 36 to 40 hr after partial hepatectomy but with a somewhat lower value than was observed in the first peak in regenerating livers not treated with hydrocortisone. In un-
reported for rat liver, kidney (6), thyroid (5), and breast (23). It now appears axiomatic that the critical initiating effects of carcinogens occur principally in proliferating cells.

Several studies using cultured rodent fibroblasts have explored whether certain phases during cell division are specifically sensitive to carcinogenesis by direct-acting methylation agents (2, 12, 19). It has been shown recently that for C3H 10T1/2 cells susceptibility to transformation by N-methyl-N’-nitro-N-nitosoguanidine rises during G1 reaching a peak at the G2-S border and falls thereafter as cells complete S and pass through G2 and M (12). Our results are consistent with a similar shift in the sensitivity of hepatocytes as they traverse the cell cycle. However, the small numbers of animals in certain treatment groups and, consequently, the small numbers of tumors actually observed preclude thorough statistical analysis of hepatocyte susceptibility as a function of cell cycle state.

In comparison to the study of Craddock and Frei (9), we observed a somewhat lower incidence of hepatocellular tumors in rats given MNU intraportally during the first peak of DNA synthesis after partial hepatectomy and a greater incidence in rats treated at 6 hr after surgery. The ages and strains of rats, the doses and routes of administration of carcinogen, and the small numbers of rats in the treatment groups in both studies can be expected to contribute to apparent differences in tumor incidences. The lack of liver tumorigenesis in rats treated at 6 hr may have been due to the greater age of the animals, as a consequence of which a smaller proportion of hepatocytes may have been in the S phase at 6 hr after partial hepatectomy (3, 11).

This study also revealed certain limitations in the use of MNU as a hepatocarcinogen. A large fraction of the dose injected intraportally clearly traversed the liver unchanged and severely complicated the experiment by inducing a high incidence of toxic changes and lethal neoplasms in nonhepatic organs. Similar results have been reported recently by others (22). This inevitably perturbed the overall physiology of the rats before causing premature death and may have indirectly affected the progression of neoplastic changes in the liver. The ideal agent would be highly specific for the liver and would be either independent of metabolism for activation (like MNU) or would be activated by mechanisms which do not change during partial hepatectomy and hepatic regeneration. Preliminary studies suggest that methyl(acetoxy)methylnitrosamine has this combination of properties when administered by intraportal injection, and experiments with this agent are currently in progress.

Finally, it is also necessary to emphasize that use of autonomous tumors as experimental end points has potential deficiencies in quantitative comparisons of this sort. When Cayama et al. (4) gave 60 mg MNU per kg at 18 hr after partial hepatectomy, they subsequently were able to detect 129 clones of initiated hepatocytes per sq cm of liver. In the present work, comparable treatment with MNU yielded at best 4 tumors in one liver and averaged less than 2 tumors/liver. This result and the fact that agents such as phenobarbital (24) exert a promoting effect on hepatocarcinogenesis and increase the tumor incidence in animals given a single dose of carcinogen after partial hepatectomy demonstrates that not all latent, potentially neoplastic liver cells induced by carcinogen exposure actually proliferate and progress to form a tumor. We do not know what proportion of latent transformed cells forms tumors under our experimental conditions, or whether the proportion is the same among hepatocytes exposed to MNU at all stages of the cell cycle. Further studies are in progress, in which an effort is made to maximize neoplastic expression by prolonged dietary administration of phenobarbital.

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REFERENCES

Inhibition of Rat Hepatocyte Proliferation by Hydrocortisone


Fig. 1. Hepatocellular carcinoma with trabecular morphology. This tumor was found in a rat given MNU 20 hr after partial hepatectomy (Group 1). Several mitotic figures can be seen in this field. H&E, × 100.

Fig. 2. Hepatocellular carcinoma, with formation of pseudoacini. This tumor was found in a hydrocortisone-treated rat given MNU 32 hr after partial hepatectomy (Group 2). H&E, × 200.

Fig. 3. Neoplastic nodule. This tumor was found in a rat given MNU 20 hr after partial hepatectomy (Group 1). Several mitotic figures can be seen in this field. H&E, × 200.
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