Growth of Carcinogen-altered Rat Hepatocytes in the Liver of Syngeneic Recipients Promoted with Phenobarbital

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ABSTRACT

An improved transplantation system for the study of carcinogen-altered hepatocytes is described. This system, which is based on that reported by Laishes and Farber (Cancer Res., 61: 507–512, 1978), involves the transfer of hepatocytes from male F344 animals to syngeneic adult hosts. Unlike the earlier protocol, the recipient rats were fed phenobarbital (PB) rather than the DNA-reactive agent, 2-acetylaminofluorene. γ-Glutamyl transpeptidase (GGT)-positive hepatocytes were induced in the donor animals by one of three different hepatocarcinogenic treatment regimens. The recipient rats received 0.05% PB in the diet for 3 wk prior to the cell transfer and were maintained on the PB diet for 2 to 7 mo.

Hepatocytes from a male F344 donor rat that had received a 70% hepatectomy and 30 mg of diethylnitrosamine per kg and had been maintained on 0.05% PB for 12 mo formed GGT-positive colonies and hepatocellular carcinomas in both male and female recipients. No GGT-positive colonies were formed when 0.05% PB was omitted from the diet of the recipients. A 70% partial hepatectomy of the recipients at the time of cell transfer was also essential for the development of colonies and tumors. The mean volume of the colonies was 5 times larger in female recipients than in males, occupying 38% of the total liver volume in the females.

GGT-positive foci arose in recipient livers that had received hepatocytes from either a male F344 donor rat treated according to the Solt and Farber [Nature (Lond.), 263: 701–703, 1976] selection protocol or a male F344 donor rat that received a 70% hepatectomy and 30 mg of diethylnitrosamine per kg and was maintained on 0.05% PB for 5 mo. The recipient animals were treated with PB which the initial experiments showed was essential for the development of foci. The number and volume of the foci in the recipient varied according to the treatment regimen that the donor rat received. This system provides a method for analyzing the growth regulation of altered foci at various stages of neoplastic development during hepatocarcinogenesis in the rat in the absence of DNA-reactive selection agents.

INTRODUCTION

By transplanting presumptive preneoplastic lesions, one is able to follow the development of specific cells and to determine the probability that they will become malignant. During the course of chemically induced hepatocarcinogenesis, a variety of enzyme-altered foci and nodules arise, and many of these lesions have been suggested as possible preneoplastic lesions (1–3). Hyperplastic nodules that are macroscopic can be dissected from the liver and transplanted. These nodules have been transplanted into mammary fat pads and spleens (4–7), where they have persisted for many months but have not developed into hepatocellular carcinomas. A preliminary report by Lee et al. (8) indicates that some nodules transplanted into the spleen do develop in metastasizing cancer. Laishes and Farber (9) developed a method for the transplantation of a suspension of isolated hepatocytes directly onto the liver. In their system, hepatocytes from a rat treated with a complete carcinogen were transplanted into rats that were fed a diet containing 0.02% acetylamino-fluorene for 1 wk prior to and for 1 wk following the transfer. The recipient animals received a 70% hepatectomy immediately preceding the transplantation. Enzyme-altered foci were apparent in the recipient rats within 10 days of the transfer, and by 17 mo hepatocellular carcinomas were present (10). Although the 2-wk feeding of 0.02% acetylamino-fluorene that the recipient rat did not induce enzyme-altered foci in the liver up to 1 wk following the end of the acetylamino-fluorene feeding (11), it is not clear what effect this carcinogen may have had on the transplanted cells. The objective of the work presented herein was to modify the Laishes-Farber protocol so that the recipient rat was never treated with a complete carcinogen. In this way, hepatocytes could be observed and analyzed for their biological potential throughout the promotion phase of development without exposure to a second dose of an initiating agent. The dependence of the growth of transplanted foci cells on the presence of the promoting agent, phenobarbital, was also tested.

MATERIALS AND METHODS

Animals. Male and female Fischer 344 rats were obtained from Harlan Sprague Dawley (Madison, WI). For Experiments 1 and 2, GGT-positive hepatocytes were induced in the donor rats according to the procedure described by Pitot et al. (12). Briefly, male rats, 160 to 180 g, received a 70% hepatectomy and 18 h later were intubated with 30 mg of DEN per kg. One wk after the intubation, 0.05% PB was added to the diet. Animals treated according to this protocol were subsequently referred to as DEN-PB donors. In Experiment 1, the donor animals were maintained on 0.05% PB diet for 53 wk, and then sacrificed. In Experiment 2, the donor animals were maintained on PB for 21 wk before being sacrificed. In Experiment 3, GGT-positive hepatocytes were induced in male rats by the treatment protocol described by Solt and Farber (13). The animals were sacrificed 4 days following the end of the 2-acetylamino-fluorene feeding. Control donor animals were prepared by feeding 0.05% PB in the diet to male rats for 3 wk prior to sacrifice.

Male (190 to 220 g) and female (140 to 170 g) rats were used as recipient animals. The recipient rats were fed a diet containing 0.05% PB for 3 wk prior to the cell transfer.

Preparation of Cell Suspension. A single cell suspension of hepatocytes was prepared by the method of Laishes and Farber (9). The liver was perfused through the portal vein with Krebs-Ringer bicarbonate buffer containing 0.2% bovine serum albumin, and the slurry was gently disaggregated by passing through a 22-gauge needle.
cytes from the donor rats was obtained by perfusing the liver with collagenase as previously described (14). The only modification was that the collagenase was dissolved in Leibovitz 15 medium containing 26 mM sodium bicarbonate (pH 7.4). Following the enzyme perfusion, the liver was dissociated in Leibovitz 15 medium. The cell suspension was passed through a sterile nylon mesh filter to remove cell clumps and tissue fragments. The cells were rinsed twice by centrifugation at 50 x g for 5 min and then resuspended in phosphate-buffered saline (4°C) at a concentration of 2.5 x 10^6 cells/ml. The cell viability was 90% to 90%. Cell smears were air dried onto glass slides and stained for GGT as described below to determine the percentage of GGT-positive cells in the suspension.

Cell Transfer Procedure. Recipient animals were anesthetized with ether. A 70% hepatectomy was performed and then the cells from the donor rat were infused into a mesenteric vein adjacent to the intestine with a 26-gauge needle as previously described (9).

In Experiment 1, the recipients were divided into seven groups. Animals in four of the groups received 2 x 10^6 cells from the DEN-PB donor, and animals in one of the groups received 2 x 10^6 cells from the control donor rats. The transfer and treatment procedure of the recipient rats was modified for each group, as shown in Chart 1, in order to analyze the importance of each component of the procedure. The recipient animals were maintained on 0.05% PB diet for 15 wk and then sacrificed.

In Experiment 2, the recipient animals received 1 x 10^6 cells. They were maintained on 0.05% PB in the diet for 28 wk and then sacrificed. In Experiment 3, the recipient animals were given injections of 2 x 10^6 donor cells per rat. The recipients were fed 0.05% PB for 8 wk and then sacrificed.

Preparation of Liver Sections. The animals were killed by decapitation. The livers were immediately removed and weighed. Two random sections were taken from each of the three lobes remaining after partial hepatectomy, positioned on filter paper, and frozen on solid CO2 as previously described (15). Serial cryostat sections, 10 µm in thickness, were cut from each frozen block. The serial sections were stained for G6Pase and ATPase according to the methods described by Zugibe (16) and were stained for GGT as described below. Prior to staining for G6Pase and ATPase, the slides were fixed for 5 min in 0.5% glutaraldehyde and were stained for GGT as described below. Prior to staining for G6Pase and ATPase, the slides were fixed for 5 min in 0.5% glutaraldehyde and then rinsed in running water for 60 s.

Histochemical Stain for GGT. Liver sections and air-dried smears of cells were stained for GGT according to the method described by Rutenberg et al. (17), with the following modifications. The concentration of the L-γ-glutamyl-4-methoxy-β-naphthylamide stock was 1.25 mg/ml and was prepared by dissolving 5 mg of L-γ-glutamyl-4-methoxy-β-naphthylamide in 0.1 ml of dimethyl sulfoxide and 0.1 ml of 1 N NaOH, then adding 3.8 ml of distilled water. The liver sections were incubated for 20 min in the staining solution.

Analysis of Foci. The GGT foci were quantitated as previously described (18). Multiple alterations of foci were analyzed in serial sections and quantitated.4

RESULTS

In Experiment 1, the treatment protocol for recipient animals was tested. The recipients were divided into seven groups and treated according to the protocols diagrammed in Chart 1. In Group A, male and female rats received control cells, which were hepatocytes isolated from a male rat that had been fed PB for 3 wk prior to the isolation. The cells from the control donor did not contain any GGT-positive cells. The livers from the recipient animals showed no GGT-positive foci (Table 1). In Group B, in which the recipients received only buffer, no GGT-positive foci were seen in the liver. In Group C, the donor cells were isolated from a DEN-PB-treated male rat that had been maintained on a PB diet for 53 wk; of the hepatocytes in the donor liver, 21% were GGT positive. Histological sections could not be taken from the animal used as the donor because the entire liver was perfused with collagenase. However, histological sections of livers from other animals treated according to the donor protocol exhibited numerous focal areas of cellular alteration, both eosinophilic and basophilic, that previous studies (19) have shown to be equivalent to enzyme-altered foci. In addition, several small neoplastic nodules were noted in some of the donor animals, but

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Table 1

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Vol. of GGT-positive foci as a % of liver vol</th>
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<tbody>
<tr>
<td></td>
<td>Males</td>
</tr>
<tr>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>8.4 ± 1.2</td>
</tr>
<tr>
<td>D</td>
<td>5.0 ± 2.9</td>
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<tr>
<td>E</td>
<td>0</td>
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<tr>
<td>F</td>
<td>0</td>
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<tr>
<td>G</td>
<td>0</td>
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4 NT, not tested.
5 Mean ± SD.

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no carcinomas were seen in the sections examined. Four mo after transplantation, large GGT-positive lesions were present in both the male and female recipients of Group C (Fig. 1). The number of lesions per liver could not be accurately determined because the lesions were too large for use in the equations that convert the data from 2-dimensional to 3-dimensional analysis. However, the GGT lesions as a percentage of liver volume could be determined directly from the 2-dimensional data. The GGT-positive lesions occupied 8.4% of the liver in the males and 38.3% of the liver in the females. In Group D, male rats received the DEN-PB cells but were not fed PB for 3 wk prior to the cell transfer. The recipients had GGT-positive lesions that occupied 5.0% of the liver. Therefore, the 3-wk PB feeding prior to the transfer had no significant effect on the formation of GGT-positive lesions. However, as seen in Groups E, F, and G, both the partial hepectomy and PB feeding after the cell transfer are essential for the development of GGT-positive lesions in the recipient.

Many of the lesions that arose in the male and female recipients were hepatocellular carcinomas. However, the growth of these cells was dependent on a partial hepectomy at the time of the transfer and on PB feeding of the recipient.

In Experiment 2, the DEN-PB donor was on a PB diet for only 4.5 mo prior to the cell isolation. One million cells from the donor were transplanted into each male and female recipient. When the recipients were sacrificed 7 mo after the cell transfer, GGT-positive foci were seen in the livers of both the male and female recipients (see Fig. 2 and Table 2). Although there are more foci in the males, the foci that are present in the females are larger than those in the males. In the female recipients, a high level of nonspecific induction of GGT was seen in some animals.

In Experiment 3, the donor animal was treated according to the Solt-Farber regimen. When these cells were transferred into recipient rats, GGT-positive foci were seen in the recipients only 2 mo after the cell transfer (Fig. 3). The number of foci was approximately the same in both the male and female recipients; however, the GGT-positive foci in the females were larger than those in the males (Table 3).

Three sets of serial sections were cut from the livers of the male and female recipients as well as from three male rats treated according to the same protocol as the donor rat used in each experiment. The sections were stained for ATPase, G6Pase, and GGT to determine the distribution of phenotypes in the liver of the donor animal and the distribution of phenotypes in the lesions that arose in the recipients. The distribution of phenotypes among the lesions was calculated by dividing the percentage of the liver having one of the seven phenotypic alterations by the percentage of the liver that was altered.

Analysis of the distribution of phenotypes showed that 65% of the altered foci in animals treated according to the Solt-Farber regimen were altered for all three phenotypes analyzed (Table 4). This finding is similar to that reported by Ogawa et al. (20). These investigators analyzed serial liver sections from animals treated according to the Solt-Farber regimen. Using a 2-dimensional analysis, they determined that 80% of the foci had increased GGT activity and decreased ATPase and G6Pase activity. However, in the recipient animals, more than three-fourths of the foci showed only a single alteration (Table 4). Some caution should be used in interpreting these data because the foci in the recipients are much smaller than the altered areas in the donors. Small foci are more difficult to see than larger ones, especially in the sections stained for ATPase and G6Pase. This difficulty may lead to an underestimation of the ATPase- and G6Pase-deficient foci and thereby lead to an underestimation of the double- and triple-altered foci. Nonetheless, these numbers may reflect a selection process in the liver. The distribution of phenotypes seen in the recipient animals in Experiment 3 is similar to the pattern found in Experiment 2 (data not shown).

**DISCUSSION**

In the transfer system reported by Laishes and Farber (9), putative preneoplastic GGT-positive hepatocytes were transferred to syngeneic recipients. The transplanted cells rapidly developed into colonies. However, the recipient animals were fed 2-acetylaminofluorene at the time of the cell transfer, and the effect of the 2-acetylaminofluorene on the putative preneoplastic cells that were transferred was unclear.

In this study donor cells, prepared by the same procedure used by Laishes and Farber, were transplanted into recipients treated only with PB. GGT-positive colonies developed in the recipient livers. Laishes and Farber saw large colonies in the recipient livers only 10 days after transplantation. The colonies in this study were analyzed 2 mo after transplantation and were smaller than those seen by Laishes and Farber. Therefore the growth of colonies was much slower in this system.

In Experiment 1, both the PB feeding and the partial hepectomy at the time of cell transfer were essential for the development of GGT-positive lesions. The donor cells in this experiment were obtained from a rat that had been fed PB for 12 mo. It is possible that small hepatocellular carcinomas were present in the liver of the donor rat. However, without a partial hepectomy or PB, these cells were unable to form colonies in the liver. The partial hepectomy may be important for two reasons. (a) It provides a growth stimulus to the liver. (b) It causes a breakdown of the intercellular junctions between hepatocytes (21), thereby facilitating the seeding of transplanted cells. These GGT-positive cells are dependent on PB for their selective growth. There were no GGT-positive cells in the livers of any of the animals that did not receive PB. PB has been shown to inhibit cell proliferation.
after partial hepatectomy (22). The role of PB in this regimen may be to inhibit cell division of the host liver selectively as is seen with the 2-acetylaminofluorene selection in the Solt-Farber protocol. Another role of PB may be to inhibit cell death (or apoptosis) during the normal turnover of liver cells. Bursch et al. (23) have provided evidence that PB promotion of clear cell and eosinophilic liver foci may be a result of inhibition of cell death in the foci and the surrounding liver. The higher rate of cell division in the foci would result in selective growth of the foci relative to the surrounding liver.

In all three experiments, GGT-positive foci arose in the recipients that received donor cells, a partial hepatectomy, and PB. Perhaps the GGT-positive foci in the donor animals should be considered malignant neoplasms, on the basis of their ability to grow (metastasize) in recipient animals. The fact that their growth is conditional on the presence of a partial hepatectomy and PB feeding is similar to growth requirements described for some transplantable tumors. Furth et al. (24) reported that the growth of transplanted primary pituitary tumors was dependent on the presence of estrogen. Both the donor cells from the experiments described here and the transplantable tumor used by Furth conform to the definition of a neoplasm suggested by Pilot (25). He defined a neoplasm as a relatively autonomous growth of tissue. It should be noted that Jirtle et al. (26) have reported that eosinophilic liver foci may be a result of inhibition of cell death in the foci and the surrounding liver.

In Experiment 3, there is a preliminary indication that the distribution of phenotypes seen in the foci that arise in the recipient is different from the distribution of phenotypes among the foci cells injected. It is known that the phenotypes of foci and carcinomas differ depending on the carcinogen used. Among foci induced by 2-acetylaminofluorene and PB, an elevated level of GGT is the most common phenotypic alteration (27). However, elevated levels of GGT are not seen in foci induced by the peroxisome proliferator Wy 14,643 (28). Recent experiments by Glauert and Pilots (33) have shown that the distribution of phenotypes may be a function of the promotion phase of the regimen. In rat liver initiated with DEN and promoted with PB, more than 80% of the enzyme-altered foci that arise are GGT positive. In rat liver initiated with DEN and promoted with Wy 14,643, the foci that arise are ATPase and G6Pase deficient but do not show elevated levels of GGT in a manner similar to that reported for hepatocellular carcinomas induced by this agent (28). The transplantation procedure described here makes possible further investigation into this phenomenon.

It is also interesting to note that, in all three experiments, the GGT-positive foci in the female recipients were larger than those in the male recipients. Both the male and female recipients were given injections of male cells. Sex differences have often been noted in the sensitivity of rats to various hepatocarcinogenic regimens (29, 30). The results reported here support earlier studies by investigators using endocrine manipulations, who concluded that the hormonal status of the animal is an important factor in its sensitivity to hepatocarcinogenesis (31). In addition, a high level of nonspecific induction of GGT was seen in some female recipients. Bone et al. (32) have reported that, following surgical manipulation, induction of GGT in periporal hepatocytes was seen to a greater extent in female F344 rats than in males.

The most important aspect of this procedure is its potential for testing the neoplastic nature of various lesions seen in a carcinogen-treated liver. With separation techniques, such as the technique for the isolation of GGT-positive hepatocytes (33), one can transplant populations enriched for specific phenotypes and determine the probability with which these cells progress to carcinomas. Furthermore, although not treated in this study, the time at which cells of hepatic foci become independent of the presence of PB may be determined in relation to the later stages of neoplastic development.

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REFERENCES

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Fig. 1. GGT-positive lesions in male and female recipients in Experiment 1. Liver sections from male (a) and female (b) rats that received a 70% hepatectomy, hepatocytes from a carcinogen-treated rat and PB feeding (Treatment Group C). A liver section from a male rat (c) that received a 70% hepatectomy and hepatocytes from a carcinogen-treated rat but no PB feeding (Treatment Group F). A liver section from a male rat (d) that received hepatocytes from a carcinogen-treated rat and PB feeding but was not administered a 70% hepatectomy (Treatment Group E).
Fig. 2. GGT-positive foci in male (a) and female (b) recipients in Experiment 2. Arrows indicate representative GGT-positive foci in the male and female livers. In the female liver, large GGT-positive foci are seen against a background of nonspecific GGT induction.
Fig. 3. GGT-positive foci in male (a) and female (b) recipients in Experiment 3. Arrows indicate representative GGT-positive foci in the male and female livers.
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