Enhanced Proliferation of Putative Preneoplastic Cells in Rat Liver following Treatment with the Tumor Promoters Phenobarbital, Hexachlorocyclohexane, Steroid Compounds, and Nafenopin

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ABSTRACT

Putative preneoplastic islands were induced in rat liver by diethylnitrosamine or nitrosomorpholine administered either as single high doses or continuously for 40 days at low dose levels. Following recovery periods of 3 weeks to 11 months, islands were identified by means of a positive γ-glutamyl transferase reaction and/or altered morphology. DNA synthesis, by means of [3H]thymidine autoradiography, as well as mitotic activity were determined. Under all conditions studied, proliferation rates of island cells were significantly higher than those of normal unaltered hepatocytes. Single doses of liver mitogens known or assumed to promote liver tumor development (phenobarbital, α-hexachlorocyclohexane, cyproterone acetate, nafenopin, and pregnenolone-16α-carbonitrile) were administered. Twenty-four to 30 hr later, this treatment produced even higher proliferative activities in island cells and increased the DNA synthesis index up to 50%, while proliferation in normal liver cells increased slightly to moderately. Thus, cells of putative preneoplastic islands appear to possess an inherent defect of growth control rendering them more susceptible to endogenous and exogenous growth stimuli. These findings partially explain why the mitogens mentioned induce rapid enlargement of preneoplastic foci and may provide a clue for further studies on the mechanism of tumor promotion in the liver. In addition, the results may form the basis for a short-term test to detect promoting activity of chemical compounds.

INTRODUCTION

Phenobarbital, some synthetic steroid sex hormones and hypolipidemic agents (e.g., clofibrate, nafenopin), as well as several environmental pollutants (e.g., α-HCH, dichlorodiphenyltrichloroethane, and butylhydroxytoluene) produce liver enlargement in rodents. This is considered to be the result of an adaptive process and is usually caused by a combination of liver cell hypertrophy and hyperplasia (21, 23). In many cases, long-term treatment of rodents with these liver mitogens results in the appearance of liver tumors (6, 18, 23, 27, 30, 31). As far as can be judged from the results of tests for mutagenicity and other tests, none of the mitogens has been shown to have an unequivocal tumor-initiating potential. In contrast, it could be demonstrated that phenobarbital (7, 11, 12, 32), sex steroids (29), dichlorodiphenyltrichloroethane (11), butylhydroxytoluene (23), α-HCH, and hypolipidemic agents (17) promote the development of liver tumors from precarcinogenic lesions. It has therefore been suggested that the appearance of liver tumors in mitogen-treated animals results from promotion of a preexisting “dormant” tumorigenic lesion of genetic or environmental origin (23, 30). However, better understanding of the underlying mechanism by which the mitogens exert their promoting activity is required for reliable safety evaluations.

The possibility of recognizing presumptive tumor cells in a preneoplastic stage (1, 2, 4, 12, 14, 20, 33) permits the study of the effects of tumor promoters on such cells. Administration of phenobarbital for several months to rats pretreated with a carcinogen was shown to result in an increase in size and number of the preneoplastic islands in the liver (7, 12). The mechanism of this effect remained unclear.

In the present study, we analyzed the effects of various liver tumor promoters on proliferation of normal and putative preneoplastic hepatocytes, the latter being identified by focal arrangement in γ-GT-positive islands (2). The results show that cells in these foci are much more sensitive to the mitogenic effects of the tumor promoters than are normal unaltered hepatocytes. These findings may provide a clue for explaining the rapid growth of preneoplastic islands induced by tumor promoters. Preliminary reports on this work have appeared elsewhere (9, 10, 26).

MATERIALS AND METHODS

Animals and Treatment. Female Wistar rats (specific-pathogen-free; Zentralinstitut für Versuchstierzucht, Hannover, Germany) were used at an initial age of 4 to 8 weeks. Three to 5 rats per cage (Macrolon) were kept under standardized conditions [22° room temperature; 50 ± 5% relative humidity; light phase, 9 a.m. to 9 p.m.; food (Altromin 1320; Lage, Germany) and water ad libitum]. Three weeks before measurements of cell proliferation, rats were set on a reversed light-dark rhythm (light phase, 9 p.m. to 9 a.m.) in which food was available for 5 hr/day only (9 a.m. to 2 p.m.). This regimen was used to synchronize hepatic cell proliferation (22, 25); it also synchronizes DNA synthesis of γ-GT-positive liver cells (9).

Single 75- or 150-mg/kg doses of DENA (Merck, Darmstadt,

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2 To whom requests for reprints should be addressed. Dedicated to Prof. Dr. Wolfgang Koransky on the occasion of his 60th birthday.
3 The abbreviations used are: α-HCH, α-hexachlorocyclohexane (α-benzene hexachloride); γ-GT, γ-glutamyl transferase; DENO, diethylnitrosamine; NNK, N-nitrosonornicotine; AAF, acetylaminofluorene; CPA, cyproterone acetate; PCN, pregnenolone-16α-carbonitrile; 3H index, percentage of [3H]-labeled cells in a cell population.
4 R. Schulte-Hermann, W. Parzefal, unpublished observations.
5 Single 75- or 150-mg/kg doses of DENA (Merck, Darmstadt,
Germany) or single 150- or 250-mg/kg doses of NNM (Serva, Heidelberg, Germany) in water were given by gavage, and measurements were made about 8 weeks later unless stated otherwise. In some studies (Charts 3 and 4), DENA (5 mg/kg) was given daily for 40 days via the drinking water; DNA synthesis was measured 3 weeks after stoppage of treatment. AAF (Merck) was administered via the diet which was prepared by saturating the pelleted food with a calculated amount of an AAF solution in acetone and subsequent drying in air at room temperature.

α-HCH (Merck), CPA (Schering, Berlin-Bergkamen, Germany), and nafenopin (Ciba-Geigy, Basel, Switzerland) were dissolved in vegetable oil; phenobarbital (Merck) was dissolved in water; PCN (Schering) was suspended in water with a trace of emulsion (Kodak, Stuttgart, Germany). The slides were exposed for 14 days and then developed with Kodak D 19B for 2 min and fixed. Nuclei were counterstained in Mayer’s hemalum for 2 min, and sections were mounted in glycerine jelly.

**Histochemistry and Autoradiography.** Fixed liver slices were embedded in Paraplast, and 3- to 5-μm sections were taken. When multiple sections were necessary to achieve a sufficiently high cell count, only every third section was used to avoid the possibility of counting the same cell twice. Subsequently, γ-GT was demonstrated in the sections by the method of Rutenberg et al. (19). Following a minimum of 1 hr in tap water, autoradiography was carried out on the same sections by the “dipping technique” using Kodak NTB photo emulsion (Kodak, Stuttgart, Germany). The slides were exposed for 14 days and then developed with Kodak D 19B for 2 min and fixed. Nuclei were counterstained in Mayer’s hemalum for 2 min, and sections were mounted in glycerine jelly.

**Slide Evaluation (Counting Technique).** Islands used for determination of proliferative activity consisted of at least 5 γ-GT-positive hepatocytes with visible nuclei and were clearly demarcated from the surrounding γ-GT-negative cells. The 3H index was determined in a minimum of 1500 normal hepatocytes or in an average of 250 (100 to 4000) cells of γ-GT-positive islands per rat. Because of the small size of the islands in most of the experiments (see “Results”), counts obtained from 10 to 30 islands in each individual liver were pooled. In addition to 3H indices, the mitotic index was determined in the 2 cell types by counting mitotic figures.

The major portion of the counting was carried out by a single observer. During counting procedures, only coded slides were used. The observer had no access to the code prior to the end of slide evaluation. A total of 65 slides were evaluated by a second observer to determine counting precision; agreement was 98.6% with respect to number of islands per section, 97% with respect to island size, and 97.7% with respect to 3H index.

**Statistical Evaluation.** Confidence limits (5) for mitotic and 3H indices were determined to establish the statistical significance of differences between normal and preneoplastic cells in individual animals. Means ± S.D. for each group are indicated in tables and charts. Intergroup differences were analyzed using Student’s t test.

**RESULTS**

**Number and Size of γ-GT-positive Islands.** γ-GT-positive liver cell islands occurred in all animals given DENA or NNM and could be found at all time points studied between 3 weeks and 1 year after application of the initiating dose. No islands were found in untreated controls (except occasionally in 1-year-old rats) or following single doses of the mitogens alone.

Islands observed 3 to 12 weeks after single doses of the carcinogens were small; at least 90% of them contained less than 20 cells/cross-section (Chart 1). Island sizes were similar in rats treated with DENA or NNM with both doses used. However, the number of islands produced [20 or 60 islands/cu mm liver following NNM (150 mg/kg) or DENA (75 mg/kg)] was increased 3- to 5-fold after administration of higher doses of NNM (250 mg/kg) or DENA (150 mg/kg). These findings are in agreement with the results of previous studies (28).

Continuous treatment with DENA (5 mg/kg daily for 40 days) produced considerably more (870/cu mm liver) and larger (average size, 44 cells/cross-section) γ-GT-positive islands than did the single doses, and island cells exhibited enhanced heterogeneity with respect to size and intensity of γ-GT staining (9).

Administration of liver mitogens had no effect on island size or number within 25 to 30 hr, but a more prolonged action of the mitogens caused a clear shift to larger islands (9). This is illustrated by the results of a representative experiment with α-HCH (Chart 1): application of this long-acting agent [biologically half-life in the female rat is about 7 days (8)] 3 weeks before measurement increased the incidence of larger islands. These

**Chart 1.** Size of γ-GT-positive islands following 1 or 2 doses of α-HCH. Rats received one dose of DENA (75 mg/kg): 1, no further treatment (n = 89); 2, 1 dose of α-HCH (200 mg/kg) 8 weeks after DENA (n = 81); 3, 2 doses of α-HCH (200 mg/kg) 5 and 8 weeks after DENA (n = 171). n, number of islands counted; 5 to 6 animals/group. Island size is expressed as cell number per island cross-section. *, p < 0.05; **, p ≤ 0.005. Bars, S.D.
results strongly suggest that the mitogens tested induce island growth.

DNA Synthesis in γ-GT-positive Islands. The frequency of DNA-synthesizing cells (3H index) was invariably higher (4- to 30-fold) in γ-GT-positive islands than in the γ-GT-negative normal liver cells, irrespective of the choice of the carcinogen, the treatment regimen, or the interval between initiation and measurement (Charts 2 and 3; Tables 1 to 4). 3H indices of γ-GT-negative normal cells were identical to those found in the livers of untreated rats of the same age, indicating that the difference in 3H indices of the 2 cell types does not result from depression of DNA synthesis of γ-GT-negative cells. 3H indices higher than normal (1.4 ± 0.5%) were found only in γ-GT-negative areas which occasionally appear in the vicinity of bile ducts; such areas can almost always be distinguished easily from γ-GT-positive preneoplastic islands.

Treatment with single doses of α-HCH and CPA resulted in an increase in the 3H index in normal hepatocytes as expected from previous work (22, 24). This effect was more pronounced in young animals (Chart 2). In γ-GT-positive islands, these 2 mitogens caused marked increases of 3H indices over the already high basal levels (Fig. 1). Following α-HCH a maximum of 30% and following CPA a maximum of 50% of these cells were synthesizing DNA (Chart 2). It is of interest to note that γ-GT-positive islands persisting for 6 or 11 months after a single dose of DENA show a response to CPA or α-HCH similar to that of younger islands. The dose of DENA used to induce island formation had no obvious effect on the response to mitogens (Chart 2).

γ-GT-positive islands induced by daily treatment with DENA (5 mg/kg) (Chart 3) or by a single dose of NNM (Table 1) may be stimulated by α-HCH in the same manner as described above. Furthermore, it should be noted that phenobarbital (Chart 3), nafenopin, and PCN (Table 2) also stimulate DNA synthesis in γ-GT-positive foci to a greater extent than in normal hepatocytes. These data suggest that the island cells respond more strongly to the mitogens tested than do normal hepatocytes.

It might be argued that the differences in the 3H indices of normal and γ-GT-positive cells were due to time shifts in the maximum levels of DNA synthesis or to an increased duration of the S phase of preneoplastic cells. These alternatives, however, appear very unlikely since the discrepancy between the 3H indices of the 2 cell types persisted after repeated injections of [3H] thymidine scheduled to label all cells replicating DNA within 18 or 24 hr (Chart 2, 7.5-month-old rats; Table 1).

As a further control, we determined 3H indices in the 2 (γ-GT-positive) cell layers immediately surrounding γ-GT-positive islands and in γ-GT-positive areas which occasionally appear in the vicinity of bile ducts; such areas can almost always be distinguished easily from γ-GT-positive preneoplastic islands. Eleven rats stimulated with α-HCH or CPA were used; a rep-
Enhanced Proliferation of Preneoplastic Hepatocytes

Mitotic Activity in γ-GT-positive Island. Mitotic activity determined after injection of colchicine was severalfold higher in γ-GT-positive foci than in normal liver cells in both unstimulated and mitogen-treated rats (Table 2). Ninety to 100% of all mitoses of both γ-GT-positive and normal cells were labeled (Table 2). Thus, the increase in mitotic activity does not result from activation of a G2 population of cells; mitosis evidently occurs as part of a regular cell cycle. The increase of mitotic activity rules out the (hypothetical) possibility that the increase of DNA synthesis as described above is due to DNA repair rather than to synthesis de novo. We conclude that γ-GT-positive island cells do indeed have a higher rate of proliferation than do normal hepatocytes and that treatment with mitogens results in a further increase in their proliferation.

DNA Synthesis in γ-GT-positive Islands of Different Sizes. The islands found after 40 days of DENA treatment were divided into different size classes, and the 3H index of these classes was calculated. Somewhat unexpectedly, large islands did not appear to have a higher 3H index than do small ones, thus indicating the same responsiveness to mitogenic stimuli (Chart 4). However, within each size class, there was a considerable variability of 3H indices found in individual islands, and the possibility cannot be ruled out that there are islands with different growth potential. Likewise, no correlation between size and proliferative activity was found in islands produced by single doses of DENA or NNM (9).

Influence of AAF on the Action of Mitogens in Normal and Island Hepatocytes. The papers of Farber (3) and Solt and Farber (28) have shown that the stimulation of DNA synthesis in normal hepatocytes subsequent to partial hepatectomy can be suppressed by low doses of AAF whereas island cells are resistant and continue to proliferate. Similarly, under our experimental conditions, AAF was found to weaken the stimulatory effect of α-HCH, CPA, and phenobarbital on normal cells, but DNA synthesis in γ-GT-positive islands was not significantly reduced (Table 3). Thus, the proliferative advantage of γ-GT-positive cells was accentuated by AAF.

DNA Synthesis in γ-GT-negative Islands. 3H indices were also determined in islands partly or completely free of histochemically detectable γ-GT activity. For this purpose, rats were used 11 months after a single dose of DENA when an appropriate number of such islands could be discovered by means of altered morphology. As shown in Table 4, about 10% of all islands found were completely γ-GT negative, and approximately 20% of islands were a mixture of γ-GT-positive and -negative cells. 3H indices of γ-GT-negative and "mixed" islands were similar to those of the γ-GT-positive islands and about 10-fold higher than in normal, unaltered hepatocytes. Interestingly, in γ-GT-positive islands without distinct morphological alterations, 3H indices were smaller than in islands exhibiting such alterations. The mitogenic effect of α-HCH was clearly present in all of the island subclasses studied (Table 4).

Table 2

<table>
<thead>
<tr>
<th>Mitogen</th>
<th>No. of animals</th>
<th>γ-GT-positive cells</th>
<th>Normal cells</th>
<th>γ-GT-positive cells</th>
<th>Normal cells</th>
<th>γ-GT-positive cells</th>
<th>Normal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6</td>
<td>1.8 ± 0.6*</td>
<td>0.1 ± 0.1</td>
<td>1.4 ± 0.3</td>
<td>0.1 ± 0.1</td>
<td>100 ± 0</td>
<td>63 ± 29</td>
</tr>
<tr>
<td>a-HCH</td>
<td>4</td>
<td>9.2 ± 1.6</td>
<td>0.9 ± 0.3</td>
<td>6.9 ± 1.9</td>
<td>0.7 ± 0.2</td>
<td>97 ± 6</td>
<td>89 ± 10</td>
</tr>
<tr>
<td>CPA</td>
<td>5</td>
<td>34.3 ± 15.9</td>
<td>9.1 ± 2.6</td>
<td>18.8 ± 8.1</td>
<td>4.3 ± 1.2</td>
<td>90 ± 8</td>
<td>93 ± 3</td>
</tr>
</tbody>
</table>

* Mean ± S.D.
Obviously, the high proliferative potential of island cells is not strictly associated with the presence of histochemically detectable γ-GT activity.

DISCUSSION

Our results show that putative preneoplastic islands of γ-GT-positive cells in rat liver proliferate at a higher rate than do the γ-GT-negative unaltered hepatocytes. Further treatment with mitogens that can promote liver tumor development leads to an even higher rate of proliferation. The enhanced proliferative activity was found independently of the experimental conditions used for induction of islands (2 different carcinogens, single or continuous treatment) and also independently of the age of animals or islands (3 weeks to 11 months). Enhanced proliferative activity of island cells did not seem necessarily associated with histochemically detectable γ-GT activity. On the other hand, occasional γ-GT-positive peripheral cells showed no increase in DNA synthesis in response to mitogens; in perinatal rat liver which exhibits high activities of γ-GT (2), α-HCH does not induce a measurable proliferative response (23, 34). Thus, enhanced proliferative activity or potential is not generally associated with the presence of increased γ-GT activities in hepatocytes but appears to be a specific characteristic of islands or foci of altered cells arising in the liver after carcinogen treatment. This conclusion is supported by the results of previous studies in which other markers such as iron exclusion and enzyme deficiencies were used for detection of islands (20, 33).

An enhanced rate of DNA synthesis was also found in islands induced by AAF and promoted by phenobarbital for 39 weeks (13), although 3H indices were much lower than in the present study. In contrast to our findings a clear difference in proliferation rate between small and large islands was noted (13). This discrepancy may possibly be due to the length of the promotion period (39 weeks), which may have resulted in a selection of islands with high proliferative potential.

Increased proliferative activity of putative preneoplastic islands (identified by ATPase deficiency) has also been noted by Rabes et al. (14, 15) but was demonstrated only in rather late stages of hepatocarcinogenesis [at 90 days or later of daily administration of DENA (5 mg/kg)]. In contrast, the present findings reveal enhanced DNA synthesis activity in γ-GT-positive islands produced by continuous exposure to DENA for only 40 days, if the carcinogen was withdrawn for 3 more weeks. Furthermore, the increased rate of DNA synthesis found in γ-GT-positive islands as early as 3 weeks after a single dose of DENA (Chart 2) provides experimental support for the hypothesis that these islands have a proliferation advantage at an early stage of development and may arise from single initiated cells (clonal genesis) (20).

A proliferation advantage of preneoplastic liver cells has been shown to exist in an antiproliferative environment created by low doses of AAF (Refs. 3 and 28; Table 3). However, our data indicate that the proliferative advantage of island cells is also present under normal physiological conditions. We see no evidence suggesting an antiproliferative action of the low levels of toxic compounds present in a normal diet or produced by bacterial metabolism that would create a "physiological" selection pressure (3) resembling the artificial situation of prolonged carcinogen feeding. Under physiological conditions, replication of normal hepatocytes presumably is in homeostasis with cell death and the demands of the body. Thus, multiplication of island cells to some degree seems to escape homeostatic controls. In addition, island cells exhibit enhanced sensitivity to mitogens eliciting liver growth which is presumably adaptive. We therefore believe that an inherent increase in responsiveness to endogenous and exogenous growth stimuli is a phenotypic characteristic of this cell type. On the other hand, the island cells analyzed in our studies share several restrictions of their growth potential with normal liver cells (9, 25); thus, they appear to be in a stage somewhere between homeostatic behavior and autonomy and may indeed be "preneoplastic" with respect to growth control.

Phenobarbital (7, 11, 32), α-HCH,4 CPA,6 and hypolipidemic agents such as nafenopin (16, 17) can be considered to be tumor promoters in rat liver on the basis of long-term studies. (PCN does not seem to have been tested for tumorigenicity; from our findings, we would predict promotional activity. This prediction awaits confirmation by appropriate animal tests.) However, the discrimination of promotion from initiation or synchroncarcinogenesis may be difficult in long-term animal studies.4 The excessive proliferation of putative preneoplastic islands in response to mitogens as described here gives more direct evidence that these agents are indeed tumor promoters. The findings do not, however, rule out the possibility that such agents may have some initiating effect in addition to their promoting activity. The increased responsiveness of putative preneoplastic cells to the mitogenic effects of the tumor promoters can at least partially explain the enlargement of islands.

Table 4  

<table>
<thead>
<tr>
<th>Mitogen</th>
<th>No. of animals</th>
<th>Normal morphology</th>
<th>Altered morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(γ-GT+)</td>
<td>γ-GT+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>γ-GT±</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>γ-GT−</td>
</tr>
<tr>
<td>None</td>
<td>5</td>
<td>0.57 ± 0.2d (45)</td>
<td>3.1 ± 1.1 (27)</td>
</tr>
<tr>
<td>α-HCH</td>
<td>7</td>
<td>2.8 ± 1.7 (37)</td>
<td>15.9 ± 9.8 (37)</td>
</tr>
</tbody>
</table>

a Criteria such as cell size, clearness or staining of cytoplasm, and size and location of nuclei were used for discrimination from normal tissue; unequivocal subclassification of all the foci observed as clear, acidophilic, or basophilic was not possible with the staining technique used (γ-GT plus hemalum); data from a few neoplastic nodules are not included.

b Islands consisting of γ-GT-positive and -negative cells.

c Mean ± S.D.

d Significantly (p < 0.01) different from 3H indices in islands with altered morphology.

* Numbers in parentheses, total number of islands found.
after short-term (Chart 1) or long-term (7) treatment and seems to provide some clue for understanding the promotion of tumor development. Stimulation of growth in normal liver seems to be a critical characteristic of the promoters used in our study (21, 23); this common feature may explain why compounds so different with respect to chemical structures and pharmacological effects all share the ability to promote liver tumor development from carcinogen-induced and possibly spontaneous tumorigenic lesions. However, it remains to be learned whether stimulation of liver growth always results in an enhanced proliferation of putative preneoplastic cells and promotion of tumor development.

Up to now, toxicological assessments of carcinogenic risks have been largely confined to the initiating ability of chemical compounds. It is, however, clear that the tumor-promoting activity of drugs and environmental pollutants is an important factor in carcinogenesis and should be taken into account in more detail. To aid the search for tumor promoters, reliable short-term tests are required that permit unequivocal discrimination between the initiating and the promoting activity of a compound. The excessive proliferative response of putative preneoplastic liver cells to promoting compounds as described here may provide the basis for such a test, but further work is required to check whether the present findings are applicable to other organs and to other species including humans.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 1. Representative micrograph with autoradiography of a γ-GT-positive island in rat liver. Rats received 1 dose of DENA (75 mg/kg); 8 weeks later, α-HCH (200 mg/kg); and, after 27 h, [3H]thymidine were given. Note the different numbers of labeled cells in the island and in the surrounding tissue. × 20.
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