Induction of Altered Hepatic Foci in Rats by the Administration of Hypolipidemic Peroxisome Proliferators Alone or following a Single Dose of Diethylnitrosamine

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ABSTRACT

The effect of feeding hypolipidemic peroxisome proliferators on the induction of altered hepatic foci (AHF) in Fischer rats was studied in order to determine whether such agents can induce or promote the development of AHF. In the first study, rats were fed ciprofibrate (10 mg/kg/day) for 1 yr. AHF, neoplastic nodules, and hepatocellular carcinomas were induced. The presence of putative γ-glutamyltranspeptidase (GGT) activity was numerically the most common marker, although it was absent in larger foci and nodules. A deficiency in canalicular ATPase and glucose-6-phosphatase provided the best markers for the larger foci and nodules. In the second study, rats were subjected to partial hepatectomy, and half of the animals were then intubated with diethylnitrosamine (10 mg/kg). One wk later, rats were fed Wy-14,643 at concentrations of 0, 0.05, and 0.1% in the diet for 6 mo. At 6 mo, the number and volume of AHF, neoplastic nodules, and hepatocellular carcinomas were induced. The presence of putative GGT activity was verified by immunohistochemical staining using an antibody to GGT. These studies show that hypolipidemic peroxisome proliferators can stimulate an increase in AHF following a single dose of diethylnitrosamine and a mitotic stimulus, and they thus can act as promoters in two-stage liver carcinogenesis. GGT is a poor marker for identifying AHF induced by peroxisome proliferators during the early, premalignant phase of hepatocarcinogenesis.

INTRODUCTION

Several hypolipidemic drugs, when fed to rodents, induce hepatomegaly and hepatic peroxisome proliferation (1, 2). If fed for extended periods of time, these agents induce hepatocellular carcinomas in rats and mice (2–5). The mechanism by which peroxisome proliferators induce hepatocellular carcinomas has been the subject of much research. Because the long-term administration of peroxisome proliferators results in the production of hepatic tumors, these agents may have both initiating and promoting activities. None of the peroxisome proliferators is strongly mutagenic in prokaryotic test systems: all peroxisome proliferators tested are negative in the Ames test (6–10). In eukaryotic test systems, peroxisome proliferators usually produce weak or negative responses (6, 8, 10–16). On the other hand, Fahl et al. (17) demonstrated that, in cell-free systems, peroxisomes isolated from livers of normal and hypolipidemic drug-treated rats induced single-strand breaks in supercoiled SV40 DNA molecules.

The role of peroxisome proliferators as promoters of hepatocarcinogenesis has been studied by several investigators (2). Several peroxisome proliferators have been found not to promote the appearance of GGT-positive foci when fed in the diet for several months (18–21). Nodules and hepatocellular carcinomas induced by the peroxisome proliferator, Wy-14,643, have additionally been found to be negative for GGT by Rao et al. (22). Schulte-Hermann and coworkers (23) have demonstrated that the peroxisomal proliferating agent, nafenopin, does increase the number of cells which are synthesizing DNA within GGT-positive foci.

The purpose of this study was to examine the effect of peroxisome proliferators on the appearance and phenotypes of AHF with three phenotypic markers: GGT; ATPase; and G6Pase. In the first experiment, ciprofibrate was fed to rats for 52 wk; in the second, Wy-14,643 was fed to rats for 6 or 14 mo after PH alone or PH followed by DEN administration. Because of the absence of GGT activity in many AHF and tumors as determined by histochemical staining, the presence of the enzyme was sought by use of a specific antibody for GGT.

MATERIALS AND METHODS

Chemicals. Diethylnitrosamine was obtained from Eastman Organic Chemicals, Rochester, NY; goat anti-rabbit IgG was obtained from Sigma Chemical Co., St. Louis, MO; ciprofibrate [2-[4-(2,3-dichloro- cyclopropyl)phenoxyl]-2-methylpropionic acid] was a gift from Sterling-Winthrop Research Institute, Rensselaer, NY; and Wy-14,643 [4-chloro-6-(2,3-xylylido)-2-pyrimidinylthio]acetic acid] was a gift from Wyeth Laboratories, Radnor, PA.

Effect of Long-Term Feeding of Ciprofibrate. Male Fischer rats (Charles River, Wilmington, MA; 80 to 100 g) were housed individually in hanging-wire, stainless steel cages and were fed a cereal-based diet. Rats were fed ciprofibrate in the diet at a dose level of 10 mg/kg/day for 52 wk. At this time, rats were killed by decapitation, and frozen liver sections were prepared as previously described (24) and then stored.

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were also stained histochemically for GGT enzyme activity by the 0.1% méthylène blue in PBS for 1 min and then dehydrated and
experiment 5 rats were fed ciprofibrate in the diet for 12 mo.

RESULTS

method of Rutenberg et al. (32) to directly compare the two procedures.

were mounted under a cover-glass.

reveal the peroxidase activity. The sections were counterstained with
for 20 min at room temperature. The slides were rinsed free of unreacted antibodies and then incubated with 3,3'-diaminobenzidine and IM >,• to

antibody to the deglycosylated GGT azoenzyme which was unreactive
with a previously titered GGT antibody used as a standard. Native

was prepared to the purified GGT by s.c. injections of a mixture of the enzyme in Freund's adjuvant into male New Zealand white rabbits.

fixed in formalin and processed for histológica! analysis. Three serial cryostat sections were prepared

sworthy et al. Briefly, tissue sections were projected onto the screen of a Talos digitizer. The Talos was connected, through a "smart-box," to a Hewlett-Packard HP9845B Graphics computer, a Hewlett-Packard HP9872C plotter, a Hewlett-Packard HP9895A flexible disc drive, and a Digital Equipment LA 120 terminal and printer. The images of the tissue sections and the focal transections were traced with a cursor on the digitizer; the information generated was fed directly into the computer. The tracings of the tissue sections and the focal transections were also drawn by the plotter. This was repeated for all 3 histochemical stains. The tissue sections were overlaid by the computer, and the number of foci per liver and the volume fraction occupied by the foci were calculated for each phenotype and for each combination of multiple phenotypes as originally described by Campbell et al. (26) and modified for the use of multiple phenotypes (27).

Effect of Feeding WY-14,643 after PH or PH/DEN. In this study rats were subjected to PH; one-half of the rats were then intubated with DEN (10 mg/kg). One wk later the rats were fed diets containing 0%, 0.05%, or 0.1% Wy-14,643. After 6 mo, rats fed either concentration of Wy-14,643 had developed significantly more foci occupying a greater volume than those fed the control diet, whether or not they received an earlier dose of DEN (Tables 1 and 2). Rats receiving DEN had the greatest number and volume of foci when fed diets with Wy-14,643. However, no dose-response was noted for the two levels of Wy-14,643 fed when either the total numbers and volume of foci was considered or when each individual phenotype was considered. In animals receiving no DEN, GGT was clearly the poorest marker of foci, while the other two markers were equally effective. Even when the animals were initiated with DEN, GGT in combination with either ATP or G6P or both was extremely infrequent.

The distribution of phenotypes is shown in Figs. 2 and 3. In rats not fed Wy-14,643, the distribution of phenotypes among the three markers, singly or in combination, was approximately equal in those that had received DEN as previously reported (33). In those animals not receiving DEN, the only significant marker noted was G6Pase. In rats fed Wy-14,643 without DEN initiation, however, relatively few foci exhibited GGT activity, and very little of the focal volume was taken up by GGT-positive foci; most of the focal volume was taken up by foci negative for ATPase or G6Pase. In those animals initiated with DEN and receiving Wy-14,643, significant numbers of GGT-positive foci were seen. However, as in the extended study with ciprofibrate (Fig. 1), the volume occupied by this phenotype group of foci was the same as or less than that occupied by the same phenotype in animals receiving DEN only with no peroxisome proliferator (Table 2; Fig. 3). Foci exhibiting a deficiency of ATPase or G6Pase, singly or in combination, occupied...
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Table 1 Effect of Wy-14,643 Treatment on Phenotype Distribution (foci/liver)

<table>
<thead>
<tr>
<th></th>
<th>Any</th>
<th>GGT</th>
<th>ATPase</th>
<th>G6Pase</th>
<th>GGT, ATPase</th>
<th>GGT, G6Pase, ATPase</th>
<th>GGT, ATPase, G6Pase</th>
</tr>
</thead>
<tbody>
<tr>
<td>No DEN Control</td>
<td>480 ± 110</td>
<td>60 ± 40</td>
<td>60 ± 30</td>
<td>410 ± 100</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>30 ± 20</td>
</tr>
<tr>
<td>0.05% Wy</td>
<td>1510 ± 320</td>
<td>60 ± 60</td>
<td>720 ± 350</td>
<td>820 ± 260</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>50 ± 20</td>
</tr>
<tr>
<td>0.1% Wy</td>
<td>1660 ± 310</td>
<td>90 ± 60</td>
<td>870 ± 140</td>
<td>960 ± 230</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>90 ± 60</td>
</tr>
<tr>
<td>+DEN Control</td>
<td>1660 ± 220</td>
<td>720 ± 150</td>
<td>480 ± 90</td>
<td>820 ± 180</td>
<td>140 ± 50</td>
<td>170 ± 70</td>
<td>150 ± 60</td>
</tr>
<tr>
<td>0.05% Wy</td>
<td>7980 ± 960</td>
<td>1200 ± 240</td>
<td>3240 ± 430</td>
<td>5100 ± 740</td>
<td>80 ± 50</td>
<td>90 ± 60</td>
<td>860 ± 150</td>
</tr>
<tr>
<td>0.1% Wy</td>
<td>5210 ± 400</td>
<td>1110 ± 200</td>
<td>2050 ± 230</td>
<td>3040 ± 240</td>
<td>40 ± 40</td>
<td>90 ± 60</td>
<td>420 ± 80</td>
</tr>
</tbody>
</table>

* Mean ± SE.
Wy, Wy-14,643.

Table 2 Effect of Wy-14,643 Treatment on Phenotype Distribution (focal volume as a percentage of liver volume)

<table>
<thead>
<tr>
<th></th>
<th>Any</th>
<th>GGT</th>
<th>ATPase</th>
<th>G6Pase</th>
<th>GGT, ATPase</th>
<th>GGT, G6Pase, ATPase</th>
<th>GGT, ATPase, G6Pase</th>
</tr>
</thead>
<tbody>
<tr>
<td>No DEN Control</td>
<td>0.030 ± 0.010</td>
<td>0.001 ± 0.001</td>
<td>0.004 ± 0.002</td>
<td>0.030 ± 0.010</td>
<td>0</td>
<td>0.003 ± 0.002</td>
<td>0</td>
</tr>
<tr>
<td>0.05% Wy</td>
<td>0.035 ± 0.008</td>
<td>0.0003 ± 0.0003</td>
<td>0.026 ± 0.006</td>
<td>0.030 ± 0.009</td>
<td>0</td>
<td>0.021 ± 0.007</td>
<td>0</td>
</tr>
<tr>
<td>0.1% Wy</td>
<td>0.087 ± 0.059</td>
<td>0.0066 ± 0.0004</td>
<td>0.073 ± 0.059</td>
<td>0.053 ± 0.039</td>
<td>0</td>
<td>0.040 ± 0.039</td>
<td>0</td>
</tr>
<tr>
<td>+DEN Control</td>
<td>0.141 ± 0.030</td>
<td>0.033 ± 0.006</td>
<td>0.033 ± 0.008</td>
<td>0.100 ± 0.027</td>
<td>0.011 ± 0.005</td>
<td>0.012 ± 0.004</td>
<td>0.012 ± 0.005</td>
</tr>
<tr>
<td>0.05% Wy</td>
<td>1.951 ± 0.798</td>
<td>0.023 ± 0.008</td>
<td>1.751 ± 0.759</td>
<td>1.640 ± 0.727</td>
<td>0.003 ± 0.002</td>
<td>0.004 ± 0.002</td>
<td>1.459 ± 0.687</td>
</tr>
<tr>
<td>0.1% Wy</td>
<td>1.361 ± 0.481</td>
<td>0.012 ± 0.004</td>
<td>1.176 ± 0.464</td>
<td>0.765 ± 0.489</td>
<td>0.0002 ± 0.0002</td>
<td>0.001 ± 0.001</td>
<td>0.592 ± 0.476</td>
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</table>

* Mean ± SE.
Wy, Wy-14,643.

All rats were subjected to partial hepatectomy; one-half of the rats were then intubated with DEN (10 mg/kg). One wk later rats were fed 0, 0.05, or 0.1% Wy-14,643 from 6 mo. The total number of foci per liver represented by GGT, ATPase, and G6Pase markers and their combinations is shown. The marker phenotype column represents the data obtained by the marker(s) listed regardless of whether or not other markers also scored the same transection.

The predominant volume of the various phenotypes and also were numerically predominant in the livers of these animals. Interestingly, combinations of these two latter markers with GGT were numerically very low and occupied almost insignificant volumes, even lower than those in animals initiated with DEN but not receiving Wy-14,643.

Histological examination of the livers of animals in this study revealed that only those animals initiated with DEN and subsequently fed diets containing the two levels of Wy-14,643 had tumors, either neoplastic nodules or hepatocellular carcinomas. At the 6-mo sacrifice, only rats at the higher dose of the peroxisome proliferator exhibited such lesions. The livers of 5 of 8 animals had neoplastic nodules, while 3 of 8 exhibited hepatocellular carcinomas. At 14 mo the livers of rats at the lower dose exhibited only neoplastic nodules (4 of 9), while at the higher dose 3 of 10 showed neoplastic nodules and/or hepatocellular carcinomas.

Immunohistochemical Studies. Sections from livers of animals fed Wy-14,643 for 6 mo following initiation by DEN/PH were examined for the presence of immunoreactive GGT with antibodies specific for the enzyme. Antibodies to the native enzyme as well as the deglycosylated enzyme were used. In Fig. 4 serial sections stained for G6Pase, GGT enzyme activity, and immunoreactive GGT can be seen. In all instances, only very small foci which do show GGT enzyme activity were detected. GGT immunoreactivity was not observed in any of the large foci of the large foci which do show GGT enzyme activity were found to contain immunoreactive GGT but at very low levels, often only barely detectable (Fig. 4E). Not all the very small GGT-positive foci were detected with the GGT antibodies, however. It is possible that some of the "GGT" activity observed in animals fed Wy-14,643 represents either an altered form of GGT or some other transferase activity which is detected with the histochemical activity stain. Interestingly, collections of lymphocytes observed in some of the Wy-14,643 animals were also positive with the activity stain but negative with both GGT antibodies.

DISCUSSION

In this study we have shown that (a) when peroxisome proliferators are fed either alone or after PH they stimulate the appearance of increased numbers of AHF, and (b) when peroxisome proliferators are fed after the administration of a single dose of DEN following PH, the number and volume of AHF are increased. This latter finding contrasts with other published work showing that the administration of peroxisome proliferators as promoters does not enhance the formation of AHF. In these studies, however, the only marker for AHF quantitated was GGT. Specifically, Numoto et al. (20) suggested that the hypolipidemic peroxisomal proliferating agent, nafenopin, actually suppressed GGT activity in both normal and focal hepatocytes. Interestingly, the data of Tables 1 and 2 support this phenomenon.
Fig. 2. Effect of feeding Wy-14,643 (W') after PH or PH/DEN on the relative frequency of phenotypes (foci per liver). All rats were subjected to PH; one-half of the rats were then intubated with DEN (10 mg/kg). One wk later rats were fed 0, 0.05, or 0.1% Wy-14,643 for 6 mo. The percentage of the total number of foci per liver represented by GGT, ATPase, and G6Pase markers and their combinations in rats subjected to PH (A) or PH/DEN (B) is shown. The marker phenotype column represents the data obtained by the marker(s) listed regardless of whether or not other markers also scored the same transection.

Fig. 3. Effect of feeding Wy-14,643 (W') after PH or PH/DEN on the relative frequency of phenotypes (percentage of total focal volume). All rats were subjected to PH; one-half of the rats were then intubated with DEN (10 mg/kg). One wk later rats were fed 0, 0.05, or 0.1% Wy-14,643 for 6 mo. The percentage of the focal volume (as a percentage of liver volume) represented by GGT, ATPase, and G6Pase markers and their combinations in rats subjected to PH (A) or PH/DEN (B) is shown. The marker phenotype column represents the data obtained by the marker(s) listed regardless of whether or not other markers also scored the same transection.

Conclusion in that the volume if not the number of GGT-positive foci decreased in animals initiated with DEN and subsequently fed Wy-14,643. However, as yet the question of GGT suppression directly by peroxisomal proliferating agents has not been answered by specific experimentation.

The present study further supports the previous finding (22) that GGT is not the most common marker for identifying peroxisome proliferator-induced AHF and tumors. In the 12-mo feeding experiment, 85% of the foci induced were positive for GGT, but these foci occupied only about 3% of the total focal volume. In the study in which Wy-14,643 was fed after PH or PH/DEN, fewer GGT-positive foci were induced than those exhibiting deficiencies of ATPase and/or G6Pase. Furthermore, GGT-positive foci present occupied only a very small fraction of the total focal volume. On the other hand, the two other markers used, ATPase and G6Pase, identified the large foci in both studies, but they did not characterize the small foci marked by GGT in the 12-mo study. Foci induced by peroxisome proliferators cannot be readily identified by using GGT as the only marker. With liver tumor promoters, such as phenobarbital or tetrachlorodibenzo-p-dioxin, GGT has been shown to be the best marker for identifying AHF (24, 33, 34); however, under other experimental conditions (35) GGT is not the predominant marker.

The lack of GGT immunoreactivity in some of the small "GGT"-positive foci suggests that these foci contain either an altered form of GGT having different antigenic determinants or a different enzymatic activity which is detected by the histochemical activity staining method. The identity of this activity is unknown at the present time. It therefore should be emphasized that GGT is not a good marker for foci from animals fed hypolipidemic peroxisome proliferators and that some of the "GGT" activity observed in these studies may not be GGT. The very light staining reactions we observed using the GGT antibodies suggest that GGT, when present in animals fed peroxisome proliferators, is present only in limited amounts. It has been recently shown that agents which increase lipid peroxidation can deplete glutathione (36). Expression of GGT may be decreased by peroxisome proliferators by chronic limitation of substrate availability.

The mechanism by which peroxisome proliferators promote
PEROXISOME PROLIFERATORS AND HEPATOCARCINOGENESIS

Fig. 4. Examination for the presence of GGT immunoreactivity in livers from rats subjected to DEN/PH and fed Wy-14,643 for 6 mo. Foci (f) in livers from rats subjected to DEN/PH and fed phenobarbital express GGT activity (A) which is efficiently detected with the native GGT antibody (B). Liver stained for G6Pase activity shows large foci (f) which are deficient in this enzyme (C). In D, a serial section from the same liver shown in C is stained for GGT activity, demonstrating lack of GGT activity in the large foci (f) but small areas which are positive for GGT (arrow). In E, a serial section from the same liver is stained for the presence of GGT immunoreactivity using a GGT antibody raised against the native enzyme. No GGT protein is present in the large foci (f), but the small GGT-positive area shows a very light staining reaction for GGT (arrow). Similar results were found using an antibody to the deglycosylated GGT enzyme (not shown). A and B, x 80; C, D, and E, x 120.

DEN-initiated GGT-negative AHF and tumors (37) is unclear. The induction of mitosis and hepatomegaly by peroxisome proliferators is consistent with their promoting properties (2). The production of reactive oxygen species by peroxisome proliferators is also consistent with their promoting ability (38, 39). The induction of peroxisomes in liver by these agents as opposed to the induction of different changes in liver by other tumor promoters may be related to the decrease in foci exhibiting GGT. The lack of GGT induction during promotion by peroxisome proliferators implies that this change is not necessary for the development of AHF and tumors. Recently De-Angelo et al. (40) found that feeding phenobarbital or a choline-deficient diet after the administration of DEN induced the phosphorylation of M, 40,000 and 175,000 plasma membrane proteins, whereas the feeding of the peroxisome proliferator did not. However, the importance of these proteins to the carcinogenic process in liver is unclear as yet.

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Induction of Altered Hepatic Foci in Rats by the Administration of Hypolipidemic Peroxisome Proliferators Alone or following a Single Dose of DiethylNitrosamine


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