Hepatocellular Carcinomas in Acatalasemic Mice Treated with Nafenopin, a Hypolipidemic Peroxisome Proliferator

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SUMMARY

The effects of long-term administration of nafenopin, a potent hypolipidemic drug with marked hepatomegalyic and peroxisome-proliferative properties, were studied in wild-type (Cs⁺ strain) and acatalasemic (Cs⁻ strain) mice. Nafenopin was administered in the diet at a concentration of 0.1% during the first 12 months and then at 0.05% until the termination of the experiment at 20 months. By 56 weeks, 100% mortality occurred in both male and female wild-type mice, whereas the mortality rate in acatalasemic mice was approximately 50%. Between 18 and 20 months of the experiment, 9 of 9 male and 12 of 12 female acatalasemic mice that survived chronic nafenopin treatment developed hepatocellular carcinomas, some of which metastasized to the lungs. None of the 15 male and 15 female acatalasemic controls developed liver cancers. Numerous peroxisomes were seen in the lung metastases of these hepatocellular carcinomas on electron microscopic examination; in contrast the number of peroxisomes in primary liver tumor cells varied considerably. The hepatocarcinogenicity of nafenopin strongly suggests the need for long-term studies with other hypolipidemic drugs that cause hepatomegaly and peroxisome proliferation to clarify the role, if any, of peroxisome proliferation in liver carcinogenesis.

INTRODUCTION

In 1965, Hess et al. (9) reported that ethyl-α-[p-chlorophenoxy]isobutyrate (clofibrate), a compound with hypolipidemic properties in man (13) as well as in several animal species (4, 14, 22), causes enlargement of the liver in male rats associated with a profound increase in the number of peroxisomes (microbodies) in liver cells. In the ensuing years, several hypolipidemic analogs of clofibrate have been found by Reddy et al. (15, 16, 19, 20) to elicit both hepatomegaly and hepatic peroxisome proliferation. Whether there is a relation between hepatic peroxisome proliferation and the hypolipidemia or these are accidental related properties of structurally related clofibrate analogs could not be ascertained. Recently, marked peroxisome proliferation was observed in liver cells of rats and mice treated with 2 novel hypolipidemic compounds that are structurally unrelated to clofibrate (18). The induction of peroxisome proliferation by these structurally unrelated compounds suggests that peroxisome proliferation is closely related to hypolipidemic property of these drugs and not to independent actions.

In view of the therapeutic importance of drugs in controlling hyperlipidemic states in man and since such individuals receive the hypolipidemic drug(s) for several years, it is necessary to ascertain the effect in animals of long-term exposure to these peroxisome proliferators. We now report that chronic administration of nafenopin (2-methyl-2-[p-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy]propionic acid; Su-13437; Chart 1), a potent hypolipidemic compound (8) with profound hepatomegalyic (3) and peroxisome-proliferative properties (16, 20), induces hepatocellular carcinomas in mice.

MATERIALS AND METHODS

Wild-type (Cs⁺ strain) and acatalasemic (Cs⁻ strain) mice (6) used in these experiments were derived from a colony maintained in this laboratory. Twenty male and 20 female wild-type and acatalasemic mice, weighing 20 to 25 g (5 to 8 weeks old), were fed nafenopin in the ground chow at a dietary concentration of 0.1% (w/w) for 1 year. At the end of 1 year, the surviving animals were fed this drug at a dietary concentration of 0.05% (w/w) until termination of the experiment at 20 months. Fifteen male and 15 female animals of each strain served as controls and were fed the same diet without the hypolipidemic drug.

Laparotomies were performed at 6, 12, 18, 26, 35, 48, 60, and 70 weeks under light methoxyflurane (Metofane; Pitman-Moore Division, Fort Washington, Pa.) anesthesia, and liver biopsies were obtained for light and electron microscopic examination and fixed and processed as described previously (16, 21). Autopsies were done when the animals were found moribund or dead. For cytochemical localization of peroxisome catalase, selected samples of liver, liver tumors, and the lung metastases from liver tumors were fixed in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate, pH 7.4 (16), and processed according to the method described by Novikoff and Goldfischer (12).

To determine the rate of hepatic DNA synthesis, [3H]thy midine (specific activity, 53.3 Ci/mmol; New England Nuclear Corporation, Boston, Mass.) was injected i.p. into 2 groups of male acatalasemic mice treated with nafenopin (0.1% w/w) for 6 and 26 weeks at a dose level of 3 μCi/g body weight and killed 60 min later. DNA was extracted from the liver and the radioactivity was determined in a Searle Mark III Spectrometer (17) and compared with appropriate controls. Serum triglyceride concentration in these...
animals was determined by the method described by Azarnoff (2).

RESULTS

There were no appreciable differences in the dietary intake or gain in body weight of wild-type (Cs\textsuperscript{a} strain) and acatalasemic (Cs\textsuperscript{b} strain) mice fed nafenopin when compared with one another or with controls. By 56 weeks, all wild-type mice treated with nafenopin died; in contrast only 11 of 20 male and 8 of 20 female acatalasemic mice died at the corresponding interval. None of the animals that died before 56 weeks developed liver tumors. The livers in these animals were markedly enlarged accounting for 20 to 26% of the body weight, whereas in control animals the liver weight constituted approximately 5 to 6% of the body weight (16). Examination with the light microscope of these livers, as well as liver biopsies obtained during laparotomy at various intervals, revealed large polygonal parenchymal cells with abundant eosinophilic granular cytoplasm and resembled "megalocytes." Liver biopsies obtained at 60 and 70 weeks from acatalasemic mice that survived long-term nafenopin administration revealed small foci of neoplastic nodules which were composed of cells with hyperbasophilic cytoplasm. In view of the limited amount of liver biopsy material that was obtained at laparotomy, a detailed account of the sequential morphological changes involved in tumor development could not be ascertained. By electron microscopy, numerous peroxisome profiles were observed in the liver cells of both male and female wild-type and in acatalasemic mice treated with nafenopin. These ultrastructural changes appeared essentially similar to those described previously (16).

Mitosis was increased in the livers of wild and catalasemic mice treated with nafenopin, particularly during the early stages of the experiment. The results of [\textsuperscript{3}H]thymidine incorporation into hepatic DNA of acatalasemic mice treated with nafenopin for 6 and 26 weeks are shown in Table 1. The data indicate that chronic administration of this compound causes significant increase in liver weight (\(p < 0.001\)) and in the total hepatic DNA, resulting from enhanced DNA synthesis. The serum triglycerides in nafenopin-treated animals were lower than that of controls.

Between 18 and 20 months of the experiment, all 9 male (100%) and all 12 female (100%) acatalasemic mice that survived chronic nafenopin administration developed liver tumors. Grossly, the liver tumors were generally multiple and varied in size from 0.3 to 3.0 cm in diameter (Fig. 1). They appeared as gray to dark brown and, on section, had a variegated pattern usually with hemorrhage and necrosis. The livers were markedly enlarged and revealed no evidence of cirrhosis or scarring. Histologically, all these tumors were well to poorly differentiated hepatocellular carcinomas (Figs. 2 to 4). Majority of these tumors were of trabecular type (Figs. 3 and 4). Several tumors were of poorly differentiated type and were composed predominantly of pleomorphic cells (Fig. 4). Mitosis was frequently noted in these tumors. Metastases in lungs were noted in 2 male and 3 female animals. These appeared as multiple, discrete, round nodules measuring 0.05 to 0.4 cm in diameter (Fig. 5, A and B). These extraparenchymal metastases were moderately differentiated and contained large polygonal cells with hyperchromatic nuclei and abundant granular eosinophilic cytoplasm (Figs. 6 and 7).

Five primary hepatocellular carcinomas and 3 metastatic tumors in the lung were studied by electron microscopy. The primary liver tumors varied in their ultrastructural differentiation but contained several peroxisomes, although their number varied considerably among tumor cells in a

![Chart 1. Structure of nafenopin (2-methyl-2-[p-1,2,3,4-tetrahydro-1-naphthyl(phenoxyl)propionic acid, Su-13437).](chart1.png)

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Serum triglycerides (mg/dl)</th>
<th>Liver wt (g/100 g body wt.)</th>
<th>Total liver DNA (mg)</th>
<th>Incorporation of [\textsuperscript{3}H]thymidine (dpm/mg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>56</td>
<td>5.3 ± 0.1</td>
<td>3.9 ± 0.27</td>
<td>25,373 ± 5,260</td>
</tr>
<tr>
<td>Nafenopin, 0.1%, 6 wk</td>
<td>4</td>
<td>19</td>
<td>16.7 ± 0.98*</td>
<td>7.2 ± 0.59</td>
<td>47,613 ± 4,580</td>
</tr>
<tr>
<td>Nafenopin, 0.1%, 26 wk</td>
<td>4</td>
<td>25</td>
<td>21.5 ± 1.30*</td>
<td>8.3 ± 0.63*</td>
<td>58,570 ± 8,766*</td>
</tr>
</tbody>
</table>

* One determination of pooled serums.
* Significantly different from controls (\(p < 0.001\)).
given tumor. Mitochondria, smooth and rough endoplasmic reticulum, and lysosomes were scanty. In the lung metastases, the tumor cells contained numerous peroxisomes which showed positive reaction (Fig. 8) when incubated in the alkaline 3,3′-diaminobenzidine medium for the cytochemical localization of peroxidatic activity of catalase (5).

The mechanism by which nafenopin exerts the carcinogenic action remains to be ascertained. It is possible that persistent mitotic activity may predispose to chromosomal abnormalities leading to neoplastic change.

From these studies it could not be ascertained if nafenopin is hepatocarcinogenic to wild-type mice, since these animals succumbed early to the dose levels used in this study. Additional studies on the dose response to nafenopin in wild-type mice would answer this question, if their survival for 18 to 20 months is achieved. Acatalasemic mice are genetic mutants derived from wild-type mice and possess markedly unstable catalase enzyme (1) and low levels of serum triglycerides (7). It is unlikely that this structural defect in catalase protein (1, 6), which is a peroxisomal enzyme, would lead to the development of liver tumors under sustained stimulus for these organelles to proliferate with nafenopin treatment. This assumption is made in view of the development of hepato cellular carcinomas in 3 inbred F-344 male rats, in our laboratory, after 18 months of treatment with 0.1% nafenopin in the diet (J. K. Reddy, unpublished data). Unpublished data also indicate that nafenopin induces liver tumors in rats (R. M. Diener, Ciba-Geigy Corp., Summit, N. J., personal communication).

Goldfischer et al. (7) reported that serum triglyceride levels are significantly lower in the acatalasemic mice than in the wild-type Cs strain. We have confirmed these observations in an earlier study (16) and showed that with nafenopin treatment the serum triglyceride concentrations are markedly lowered. The serum triglyceride levels in acatalasemic mice after 6 and 26 weeks of nafenopin treatment in this study are significantly lower (Table 1). The increase in peroxisome population in liver cells and the associated increase in hepatic cataloperoxidase (7) may be responsible for further lowering of serum triglycerides with nafenopin treatment. The role, if any, of hepatic peroxisome proliferation induced by several hypolipidemic drugs (18) in lowering serum cholesterol and serum triglyceride levels is not clear. However, it is now increasingly evident that all potent hypolipidemic compounds possess hepatic peroxisome proliferative property, suggesting a relation between peroxisome proliferation and hypolipidemia (18).

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REFERENCES


**Figs. 2 to 4, 6 and 7. All sections are stained with H & E.**

**Fig. 1.** Multiple liver tumors in a male acatalasemic mouse killed at 18 months. Nafenopin content was 0.1% in the diet for 12 months and 0.05% for additional 6 months.

**Figs. 2 and 3.** Trabecular hepatocellular carcinomas from a female acatalasemic mouse on nafenopin for 19 months (Fig. 2) and a male mouse on nafenopin for 20 months (Fig. 3). Fig. 2, × 120. Fig. 3, × 160.

**Fig. 4.** Poorly differentiated hepatocellular carcinoma from a female mouse treated with nafenopin for 19 months. × 140.

**Fig. 5.** Numerous discrete metastases in the lungs from a hepatocellular carcinoma of a female acatalasemic mouse treated with nafenopin for 20 months. Anterior (A) and posterior (B) views. × 40.

**Fig. 6.** Multiple metastases in the lungs from a poorly differentiated hepatocellular carcinoma. × 10.

**Fig. 7.** Higher magnification of a metastatic hepatocellular carcinoma in the lung. The tumor cells display nuclear pleomorphism and contain abundant granular eosinophilic cytoplasm. × 160.

**Fig. 8.** Portion of a tumor cell from lung metastases of a hepatocellular carcinoma. The glutaraldehyde-fixed tumor tissue was incubated in alkaline 3,3'-diaminobenzidine medium for cytochemical localization of peroxisome catalase. Note the presence of reaction product in peroxisomes (microbodies) which are markedly increased in number. There is also some increase in mitochondrial number. × 18,000. Inset, 0.5-µm-thick section of metastatic tumor in lung incubated for peroxidase activity and photographed with phase optics. × 1200.
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