Excessive Accumulation of Autofluorescent Lipofuscin in the Liver during Hepatocarcinogenesis by Methyl Clofenapate and Other Hypolipidemic Peroxisome Proliferators

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ABSTRACT

Several hypolipidemic drugs and certain industrial plasticizers induce proliferation of peroxisomes and enhance the activities of peroxisome-associated enzymes in the livers of rodents. Evidence now suggests that potent hepatic peroxisome proliferators as a class are carcinogenic, although they do not appear to be mutagenic in the Salmonella-microsome assay system. We now report that long-term administration of methyl-2-[4-(p-chlorophenyl)phenoxy]-2-methylpropionate, a potent hepatic peroxisome proliferator, at a dietary concentration of 0.1% (w/w), induced hepatocellular carcinomas in 14 of 14 male F344 rats between 65 and 75 weeks. The tumor cells contained several peroxisomes. Consistent with this observation was the finding of increased levels of carnitine acetyltransferase, heat-labile peroxisomal enoyl coenzyme hydratase, and peroxisomal β-oxidation system in the tumors.

As expected, methyl clofenapate was not mutagenic in the Salmonella-microsome assay using Salmonella typhimurium strains TA98 and TA100. Abundant accumulation of autofluorescent lipofuscin in the nontumorous portions of liver in rats bearing methyl-2-[4-(p-chlorophenyl)phenoxy]-2-methylpropionate-induced hepatocellular carcinomas was observed. The examination in this study of livers of rats bearing hepatocellular carcinomas induced previously by five other hypolipidemic peroxisome proliferators provided retrospective evidence for increased accumulation of autofluorescent pigment in the liver cells. It is suggested that accumulation of lipofuscin in the livers of rats fed peroxisome proliferators serves as evidence for the increased production of biologically damaging free radicals as a result of H2O2 generated by sustained proliferation of peroxisomes. The hypothesis that persistent proliferation of peroxisomes and increase in peroxisomal β-oxidation system serves as endogenous initiator of the neoplastic transformation of liver cells by increasing the intracellular production of DNA-damaging H2O2 and other reactive oxygen intermediates (OH−, O2•−, 1O2) remains to be tested.

INTRODUCTION

It is now well established that a variety of structurally diverse chemicals with hypolipidemic property cause hepatomegaly and hepatic peroxisome proliferation in rats and mice (9, 25, 26, 28, 42). The identification of the fatty acid β-oxidation system in rat liver peroxisomes (14, 16) and its enhancement in the liver and kidney of rodents fed different peroxisome proliferators (10, 12, 14, 16, 23, 38) support our suggestion that drug-induced proliferation of peroxisomes is related to lipid metabolism (28). Recent studies have shown that the widely used hypolipidemic drug clofibrate6 and 4 other newly developed hypolipidemic agents with hepatic peroxisome proliferative property induce hepatocellular carcinomas in rats and/or mice (27, 31, 32, 34, 35, 41). This led to the suggestion that potent hepatic peroxisome proliferators as a class are carcinogenic (27). In support of this hypothesis are the positive results of a recent carcinogenesis bioassay in rats and mice of di(2-ethylhexyl)phthalate (6), an extensively used industrial plasticizer (7) which also exhibits serum lipid lowering and hepatic peroxisome proliferative properties (2, 20, 30).

The implication that hepatic peroxisome proliferation induced by hypolipidemic drugs and industrial plasticizers leads to the development of liver tumors is serious because of the widespread concern over the therapeutic and environmental safety, respectively, of these 2 divergent groups of chemicals. Continued surveillance of humans on hypolipidemic drug therapy as well as evaluation of all new drugs in animals for possible adverse effects appear necessary in order to gain additional insights into the toxicity mechanisms. The present study deals with the effects of long-term administration in rats of methyl clofenapate (Chart 1), a very potent lipid-lowering agent which is structurally related to clofibrate (46). We report that methyl clofenapate induces hepatocellular carcinomas in rats and leads to an excessive accumulation of autofluorescent lipofuscin in the liver. The present study also includes retrospective analyses of livers of rats bearing hepatocellular carcinomas induced by 5 other hypolipidemic peroxisome proliferators for lipofuscin accumulation. The possibility that lipofuscin accumulation may represent evidence, although indirect, for sustained increase in the intrahepatic production of oxygen radicals as a result of persistent peroxisome proliferation is considered.

MATERIALS AND METHODS

Salmonella-Microsome Assay. The mutagenic activity of methyl clofenapate was tested using the Salmonella/microsome assay (1, 48). S. typhimurium strains TA98 and TA100 were obtained from Professor B. N. Ames, University of California, Berkeley, Calif. The experiment included solvent controls as well as a known direct-acting mutagen (sodium azide) and a mutagen that requires metabolic activation (2-acetylaminofluorene).

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Liver Tumor Induction by Methyl Clofenapate. The pattern of death and the liver tumor incidence in male F344 rats fed methyl clofenapate in the chow are shown in Table 2. A consumption of 12 to 15 mg methyl clofenapate per day per rat (~100 mg/week) was estimated over the 75-week experimental period. Between 85 and 75 weeks, 14 of 14 rats on methyl clofenapate treatment developed multiple liver tumors, whereas no tumors were found in rats fed the control diet. The liver tumors were multiple and presented as gray-brown nodules approximately 2 to 45 mm in diameter. The uninvolved liver parenchyma of these tumor-bearing rats displayed an intense green-brown color. These livers were markedly enlarged. Histologically, the liver tumors were hepatocellular carcinomas with well-to moderately differentiated trabecular patterns. The tumor cells were polyhedral with abundant granular cytoplasm. They contained very large nucleoli. Mitotic activity was prominent. Metastases in lungs were found in 3 animals, which presented as multifocal microscopic cell clusters of hepatocellular carcinomas. The ultrastructural characteristics of the methyl clofenapate-induced hepatocellular carcinomas were similar to the overall features of liver cell tumors induced by other chemical carcinogens (43), except that many tumor cells contained prominent peroxisomes (Fig. 1). Peroxisomes were, however, fewer in number in the cells of larger tumors.

Peroxisomal Enzymes in Rats Bearing Methyl Clofenapate-induced Primary Liver Tumors. Because these rats were fed the diet containing methyl clofenapate until termination of the experiment, it appeared pertinent to examine the levels of peroxisome-associated enzymes in primary hepatocellular car-

### Table 2

<table>
<thead>
<tr>
<th>No. of rats alive at</th>
<th>No. of rats with hepatocellular carcinomas</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Methyl clofenapate</td>
<td>14</td>
</tr>
</tbody>
</table>

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**Absence of Mutagenicity of Methyl Clofenapate.** Methyl clofenapate, over a wide concentration range, when tested in the absence or presence of rat liver S9 microsomal mixture, failed to induce a dose-dependent increase in the number of histidine-positive revertant colonies with S. typhimurium strains TA98 and TA100 (Table 1). It is concluded that methyl clofenapate, like other hypolipidemic drugs (48), is not mutagenic in this test system.
cinosas and the surrounding nontumor liver. The results presented in Table 3 show a significant elevation in the activities of carnitine acetyltransferase and peroxisomal enoyl-CoA hydratase in the tumors and nontumorous portions of liver when compared to normal rat liver. The peroxisomal fatty acid β-oxidation capacity was also increased in the liver and liver tumors of methyl clofenapate-fed rats. The catalase activity in the liver tumors, however, was substantially lower than that of normal liver, despite the presence of several peroxisomes in these tumor cells. This indicates that, although other peroxisomal enzymes are inducible in liver tumors, catalase is not.

Induction of a M.W. 80,000 Peroxisome Proliferation-associated Polypeptide in Tumor-bearing Rats. The SDS-polyacrylamide gel electrophoretic patterns of large particle and microsomal fractions obtained from the livers of normal rats and from the liver and primary hepatocellular carcinomas of rats fed on methyl clofenapate are illustrated in Fig. 2. The fractions obtained from the nontumorous portions of liver of methyl clofenapate-treated rats showed a marked increase in the content of a M.W. 80,000 protein which is associated with peroxisome proliferation (29, 37). The amount of this protein is also increased in the fractions obtained from the liver tumors, suggesting that the tumor cells respond to the peroxisome proliferative effect of methyl clofenapate.

Lipofuscin Accumulation in the Livers of Rats on Long-Term Peroxisome Proliferator Feeding. The light and electron microscopic examination of the uninvolved liver in rats bearing methyl clofenapate-induced primary hepatocellular carcinomas revealed excessive accumulation of lipofuscin (Figs. 3 to 5). When hematoxylin-and-eosin-stained sections were examined by light microscope, the pigment appeared as yellow-brown granular material. Although the pigment appeared abundant in the peribiliary location, granules of various sizes were found throughout the hepatocyte cytoplasm. Some of these pigment granules were as large as the hepatocyte nuclei (Fig. 3, inset). By electron microscopy, the lipofuscin granules appeared as polymorphic material within lysosomes (residual bodies). These lysosomes were characterized by a single limiting membrane; their contents were obscured, in most part, by the presence of pigment and other indigestible debris (Figs. 3 to 5). The larger lysosomes displayed many lipid droplets and, at times, elongated cholesterol-clefts within their matrix (Figs. 3 and 4).

Because lipofuscin represents highly oxidized lipid, which is capable of emitting a bright yellow-green to orange fluorescence when examined with UV (44, 45), it was relatively easy to determine the extent of lipofuscin accumulation in the livers of rats bearing methyl chlofenapate-induced hepatocellular carcinomas through this fluorescence (Figs. 6 and 7). The nontumorous portions of liver of all methyl clofenapate-fed rats showed a marked increase in the extent of granular autofluorescence. The hepatocellular carcinoma cells contained only an occasional fluorescent granule. The relatively low amount of reduced autofluorescent pigment in the tumor is most likely due to increased cell proliferation and mitosis in neoplastic cells. It is conceivable that cells dividing actively discharge their content of indigestible pigment during cytokinesis.

To determine whether excessive accumulation of lipofuscin in liver cells is a phenomenon associated with peroxisome proliferator-induced hepatocarcinogenesis, we examined the distribution of lipofuscin by autofluorescence in the livers of rats bearing primary hepatocellular carcinomas induced previously in our laboratory by 5 other peroxisome proliferators (Table 4). An excessive amount of lipofuscin was found in the livers of all rats fed these peroxisome proliferators. 4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthio(3-β-hydroxyethyl)acetamide, 2-chloro-5-(3,5-dimethylpipеридиносульфонил)бензойная кислота (Fig. 8), 2-methyl-2-(1,2,3,4-tetrahydro-1-naphthyl)phenoxypropiolic acid, and WY-14,643 appeared to cause as much accumulation of lipofuscin as did methyl clofenapate. In contrast, the livers of 14- to 28-month-old control rats (Fig. 9) and livers of rats bearing tumors induced by aflatoxin B1 or the combination of lasiocarpine and thioacetamide failed to display an appreciable amount of fluorescence.

**DISCUSSION**

The hepatocarcinogenicity in F344 rats of methyl clofenapate described here should be considered together with results obtained with other hepatic peroxisome proliferators which have been tested previously (27, 31, 32, 34, 35, 41). Methyl clofenapate, a closely related structural analog of clofibrate (46), is several times more effective in inducing hepatomegaly and peroxisome proliferation in rats and mice (25). The observation that long-term administration of methyl clofenapate leads to the development of hepatocellular carcinomas in rats is consistent with the hypothesis that chemicals capable of inducing hepatic peroxisome proliferation in rodents are carcinogenic (27). In the present study, methyl clofenapate was not found to be mutagenic when tested with 2 strains of _S. typhiurium_ (TA98 and TA100) that are highly sensitive to mutagenic chemicals. The absence of mutagenicity of methyl clo-

<table>
<thead>
<tr>
<th>Group</th>
<th>Catalase (μmol/min/mg protein)</th>
<th>Carnitine acetyltransferase (μmol/min/mg protein)</th>
<th>Heat-labile enoyl-CoA hydration (μmol/min/mg protein)</th>
<th>1-14C]Palmitoyl-CoA oxidation (μmol/min/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor-bearing rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatocellular carcinomas</td>
<td>30 ± 6a</td>
<td>118 ± 24a</td>
<td>11 ± 3.1b</td>
<td>5.50 ± 1.31b</td>
</tr>
<tr>
<td>Uninvolved liver</td>
<td>66 ± 8a</td>
<td>103 ± 18b</td>
<td>23 ± 6.5b</td>
<td>9.44 ± 0.76b</td>
</tr>
<tr>
<td>Normal rat</td>
<td>40 ± 4</td>
<td>4.5 ± 0.9</td>
<td>2.2 ± 0.8</td>
<td>1.14 ± 0.27</td>
</tr>
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*a* Mean ± S.D. of 4 to 8 animals in each group.

*b* Significantly different from normal rats (p < 0.005).
The livers of rats bearing hepatocellular carcinomas induced by methyl clofenapate in the present study and by other peroxisome proliferators reported previously have been examined for lipofuscin. Tumors induced by aflatoxin B1, and by lasiocarpine and thioacetamide are from an unpublished study. Unstained deparaffinized sections (3 μm thick) were examined for yellow-orange autofluorescence by a Leitz fluorescence microscope. Results are expressed as: ±, focal spotty fluorescence; +, scattered periportal fluorescence limited mostly to the centrolobular zone; ++, periportal involvement of the entire liver lobule (diffuse peribiliary); ++++, generalized involvement of the hepatocyte cytoplasm including perinuclear clustering; and +++++, generalized parenchymal with colonies of intensely fluorescent hepatocytes.

There is increasing evidence to show that hypolipidemic hepatic peroxisome proliferators fail to interact with and damage cellular DNA (48). Warren et al. (48) reported that several carcinogenic hypolipidemic drugs, such as clofibrate, 4-chloro-6-(2,3-xylidino)-2-pyrindiminothio(N-B-hydroxyethyl)acetamide, 2-methyl-2-(p-(1,2,3,4-tetrahydro-1-naphthyl)phenoxyl)propanic acid; BR-931 (pyrinixil), 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio(N'-/β-hydroxyethyl)acetamide; tibric acid, 2-chloro-5-(3,5-dimethylpiperidinosulphonyl)benzoic acid. Because DNA damage is generally believed to be the initial biochemical alteration leading to the neoplastic transformation (initiation), it is widely held that such a genetic alteration is the result of interaction with DNA of a carcinogenic chemical, either directly or after metabolic activation to electrophilic reactants (1, 8, 19). Therefore, the absence of mutagenicity of methyl clofenapate and other hypolipidemic drugs under the conditions tested raises the question as to the mechanism by which these chemicals with peroxisome proliferative property induce liver tumors in rats and mice. The finding of a strong positive correlation between hepatic peroxisome proliferation and the development of hepatocellular carcinomas prompted Reddy et al. (27) to postulate that the peroxisome proliferator liver carcinogenesis is linked to the metabolic disturbance emanating from the sustained increase in hepatic peroxisome population.

There are increasing evidence to show that hypolipidemic drugs and the industrial plasticizers that induce hepatic peroxisome proliferation in rodents consistently increase the peroxisomal β-oxidation system (10, 12, 14, 16, 23, 38) and induce a M.W. 80,000 polypeptide which is localized in the matrix of peroxisomes (29, 37). In the present study, a significant increase in the activity of the peroxisomal fatty acid oxidizing system was found in both the tumor and nontumorous portions of liver in methyl clofenapate-fed rats. Similar enhancement of this enzyme system was also found in the livers of rats bearing liver tumors induced by another peroxisome proliferator, Wy-14,643 (13). A 3- to 15-fold increase in peroxisomal β-oxidation of fatty acids appears to cause an excess generation of H2O2 (10). This is because the first dehydrogenation step in peroxisomal β-oxidation involves the reduction of O2 to H2O2 due to the flavin adenine dinucleotide-dependent fatty acyl-CoA oxidase (14). Peroxisomal catalase is one of the cellular defense mechanisms responsible for destroying most of the H2O2 formed in this organelle (4). According to Poole's calculations, about 2% of H2O2 generated within the peroxisome in vivo in normal liver diffuse out (24). In contrast, in isolated peroxisomal preparations, between 11 and 42% of the H2O2 generated diffused into the external medium (3), suggesting that alterations in peroxisome membrane can lead to excessive escape of H2O2. It is conceivable that sustained increase in peroxisome proliferation can lead to alterations in peroxisomal membrane permeability to H2O2. Such an assumption is not unreasonable since it is already known that proliferated peroxisomes are abnormal with regards to their biochemical composition (17, 25). The 2-fold increase in catalase synthesis in the livers with peroxisome proliferation (28) is disproportionately less than the increase in peroxisome volume and the H2O2-generating peroxisomal β-oxidation enzyme system (12, 14). The H2O2 diffusing out of proliferated peroxisomes may lead to the production of another free radical (OH-) in the presence of Fe2+ by the Haber-Weiss reaction (4). Preliminary studies indicate that there is an excess amount of H2O2 in the livers of rats bearing hepatocellular carcinomas induced by the peroxisome proliferator Wy-14,643 (13). The relative rates of H2O2 generation in the livers of animals fed different peroxisome proliferators, therefore, need to be correlated with their potency to induce hepatic peroxisome proliferation, alterations in membrane permeability, and hepatocellular carcinomas. Furthermore, it would be important to determine the levels of potential DNA-damaging oxygen radicals such as H2O2 (47), O2·, and OH· (39) during peroxisome proliferator-induced hepatocarcinogenesis.

The finding that long-term administration of methyl clofenapate leads to an excess accumulation of yellow autofluorescent lipofuscin in the liver in the present studies is construed as evidence, although indirect, for sustained increase in the intracellular production of DNA-damaging oxygen radicals and by inference for the initiation of neoplastic change by the metabolic by-products of peroxisomes. The exact nature of the fluorescent chromophore(s) in lipofuscin is poorly defined, but the fluorescence is generally attributed to products of lipid peroxidation. For example, nd N,N'-disubstituted 1-amino-3-iminoprene, R—N=CH—CH=CH—NH—R, resulting from the reaction of malondialdehyde with amino compounds (possibly with amines, RNA, DNA, and phospholipids) has been shown to possess fluorescence properties resembling those of lipofuscin (44). Wolfe et al. (49) identified retinoyl complexes as the autofluorescent component of the neuronal lipofuscin stored in Batten disease. To what extent, if any, retinoids contribute to the autofluorescence in the lipofuscin stored in the liver parenchymal cells of peroxisome proliferator-treated...
rats remains to be determined. The examination in this study of
livers of rats bearing hepatocellular carcinomas induced by
other peroxisome proliferators provides retrospective evidence
of increased accumulation of autofluorescent pigment (Table
4). No such lipofuscin accumulation was encountered in the
livers of control rats and rats bearing liver tumors induced by
the chemical carcinogens aflatoxin B1 and lasiocarpine which
do not appreciably increase the liver peroxisome population.
In this regard, it is pertinent to note that excess lipofuscin
accumulates in the tissues of animals fed diets deficient in the
antioxidant α-tocopherol (vitamin E) and selenium (45); whether
this reflects free radical damage or the inability of hepatocel-
lar lysosomes to breakdown sequestered materials or both is
clear. It is also of importance to point out that rats fed a
vitamin E-deficient diet show increased peroxisomal enzyme
activity in their livers (33). It is tempting, therefore, to suggest
that accumulation of lipofuscin in the liver cells of rats fed
peroxisome proliferators is secondary to the increased produc-
tion of biologically damaging free radicals as a result of \( \text{H}_2\text{O}_2 \)
generated by persistent increase in peroxisomal \( \beta \)-oxidation
of fatty acids. It is further suggested that a DNA injury, if essential
for the initiating event in peroxisome proliferator-induced can-
cer, may be caused by continued oxygen radical toxicity re-
sulting from excessive generation of \( \text{H}_2\text{O}_2 \) which ensues sus-
tained elevation of peroxisomal fatty acid \( \beta \)-oxidation system
(36). The findings of increased peroxisomal \( \beta \)-oxidation system,
 excess accumulation of lipofuscin, increased concentra-
tion of hepatic \( \text{H}_2\text{O}_2 \), and lack of mutagenicity of these agents
are pointing to the role of continued peroxisome proliferation
in carcinogenesis following the long-term administration of
peroxisome proliferators.

Proliferation of peroxisomes and the development of hepato-
cellular carcinomas resulting from the administration of hy-
polipidemic chemicals are touted by some to be specific to the
rodent species and therefore of no consequence to humans
(5). Such conclusions may be unwarranted since no data are
available on the effects of long-term administration of these
agents, over a wide dose range, on the peroxisomal enzymes
and liver tumor development in nonrodent species. Such infor-
mation is vital before it can be argued that the finding of
hepatocellular carcinomas in rodent species treated with hy-
polipidemic agents has no known relevance to the risk of
development of liver tumors in humans (5). In contrast, the
recent follow-up study of the WHO cooperative trial on primary
prevention of ischemic heart disease, using clofibrate to lower
serum cholesterol, has demonstrated that there were 25% more
deaths (including those from cancer and ischemic heart
disease) in the clofibrate-treated group than in the comparable
serum cholesterol, has demonstrated that there were 25%
more deaths (including those from cancer and ischemic heart
disease) in the clofibrate-treated group than in the comparable
control group (22). The reasons for the
increased mortality in the clofibrate-treated group in the WHO
trial are not evident. However, these adverse effects of long-
term administration of hypolipidemic drugs observed in humans
and rodents should be considered carefully until we understand
the underlying mechanisms.

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Fig. 1. Methyl clofenapate-induced hepatocellular carcinoma. The tumor cells contain many peroxisomes (p.). × 7,500.

Fig. 2. SDS-polyacrylamide gel electrophoretic profile of large particle (Lanes 1, 3, and 5) and microsomal (Lanes 2, 4, and 6) fractions obtained from normal rat liver (Lanes 1 and 2); uninvolved liver of a rat-bearing methyl clofenapate-induced tumor (Lanes 3 and 4) and primary hepatocellular carcinoma developed in a rat fed methyl clofenapate (Lanes 5 and 6). Protein concentration was 20 µg/lane. The arrow indicates the position of the M.W. 80,000 peroxisome proliferation-associated polypeptide.

Fig. 3. Lipofuscin (Lc) within lysosomes in the nontumorous liver parenchymal cells in a rat bearing methyl clofenapate-induced hepatocellular carcinomas. These granules correspond to the large cytoplasmic inclusions observed by light microscope (inset; arrow). Several peroxisomes (P) are also evident in the electron micrograph. × 8,000; inset, × 480.

Fig. 4. Large and small lipofuscin (Lc) granules are evident in the nonneoplastic liver cells in the methyl clofenapate-fed rat. Note the presence of numerous peroxisomes. × 13,500.

Fig. 5. Numerous lipofuscin (Lc) granules are distributed throughout the cytoplasm in the liver cell of a rat bearing methyl clofenapate-induced hepatocellular carcinoma. × 13,500.

Figs. 6 to 9. Unstained deparaffinized sections examined for autofluorescence. In these black and white photographs, the yellow-orange fluorescence of lipofuscin appears as white.

Fig. 6. Diffuse peribiliary fluorescence involves the entire liver lobule in a rat bearing hepatocellular carcinoma induced by methyl clofenapate. Arrows, large granules the ultrastructure of which is represented in Figs. 5 and 6. × 375.

Fig. 7. Intense cytoplasmic fluorescence in the nontumorous portion of liver in a methyl clofenapate-fed rat. The hepatocellular carcinoma cells (HC) reveal scant fluorescence. × 375.

Fig. 8. Diffuse cytoplasmic fluorescence in the nontumorous portion of liver in rat fed thiotic acid. Note the absence of granular fluorescence in the tumor cells (HC). × 520.

Fig. 9. Liver of a normal rat aged 24 months. Note the absence of granular fluorescence. × 540.
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