Concomitant Suppression of Hyperlipidemia and Intestinal Polyp Formation in Apc-deficient Mice by Peroxisome Proliferator-activated Receptor Ligands

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ABSTRACT

Epidemiological studies have shown a positive association of colon cancer with hyperlipidemia. Furthermore, signaling generated by peroxisome proliferator-activated receptor (PPAR) α and γ ligands, suggested to be candidate tumor preventive agents, has been shown to lower serum triglyceride levels. In the present study, we assessed hyperlipidemia in Apc-deficient mice, model animals for human familial adenomatous polyposis, and examined the effects of pioglitazone and bezafibrate, respectively, PPARγ and PPARα agonists, on both hyperlipidemia and intestinal polyposis. Serum lipid levels in Apc1309 mice and Min mice from 6 to 15 weeks of age were measured. Although serum levels of triglyceride and cholesterol were low in both Apc1309 and wild-type mice at 6 weeks, triglycerides were elevated 10-fold in Apc1309 mice by the age of 12 weeks but not in their wild-type counterparts. Cholesterol was also increased significantly, and marked centrifibular-restricted steatosis was observed in the livers of aged Apc1309 mice. Similar findings were observed for Min mice at 15 weeks of age. Moreover, lipoprotein lipase mRNA levels in the liver and small intestine of Apc1309 and Min mice were demonstrated to be lower than those in wild-type mice. Treatment of Apc1309 mice with 100 and 200 ppm pioglitazone or bezafibrate for 6 weeks of age caused dose-dependent reduction in serum triglycerides and cholesterol, along with a reduction in the numbers of intestinal polyps to 67% of the control value. The present study clearly demonstrated a hyperlipidemic state in Apc gene-deficient mice and a potential of PPARα and PPARγ ligands to suppress both hyperlipidemia and polyp formation. Hyperlipidemia in these mice may thus be associated with their intestinal lesion development.

INTRODUCTION

The risk of colon cancer appears to be elevated by a high fat diet (1), and epidemiological studies have shown a clear association with serum triglycerides and cholesterol (2, 3). It has been reported that reduction of cholesterol levels by HMG-CoA reductase inhibitors can reduce 7,12-dimethylbenz(a)anthracene-induced mammary gland tumor development in rats (4).

PPARs are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily (5, 6). Three PPAR isotypes have been identified: α, δ (β), and γ. PPARγ is highly expressed in fat tissue with important roles in adipocyte differentiation and lipid storage (7). PPARγ is also expressed in a number of epithelial neoplasms, including examples in the colon, breast, and prostate (6). PPARγ ligand thiazolidinediones, including troglitazone, and the tyrosine analogue GW7845 can induce apoptosis and adipogenic differentiation and inhibit tumor growth both in vitro and in vivo (8–10). It is further known that the PPARγ ligand pioglitazone, another thiazolidinedione, inhibits the growth of human renal cell carcinoma, hepatocellular carcinoma, gastric cancer, and salivary gland cancer cells in vitro (11–13). PPARα is predominantly expressed in liver, heart, kidney, intestinal mucosa, and brown adipose tissue, all with high catabolic rates of fatty acids and peroxisomal metabolism (5), and a PPARγ ligand, Wy-14,643, is reported to reduce 7,12-dimethylbenz(a)anthracene-induced mammary gland tumor development in rats (15).

Pioglitazone is a potent PPARγ agonist and a weak PPARα agonist, and bezafibrate is a specific PPARα agonist (16). These ligands improve hypertriglyceridemia and hypercholesterolemia via induction of adipocyte-specific genes, such as LPL (17). Recently, it was reported that 100 and 200 ppm pioglitazone, bezafibrate, or troglitazone in the diet can suppress formation of dextran sodium sulfate/AOM-induced ACF, putative preneoplastic lesions, in the rat colon (18). On the other hand, it has been reported that high doses of troglitazone and rosiglitazone promote polyp formation in the Min mouse colon (19, 20).

The Apc1309 (C57BL/6J/Apc1309) mouse, an animal model of human FAP, develops numerous polyps in the intestinal tract because of a truncation mutation in the adenomatous polyposis coli (Apc) gene (Apc1309, Ref. 21). It is considered to have advantages for investigations of intestinal carcinogenesis and evaluation of anticancer and chemopreventive agents, as with other FAP model mice, such as ApcMin (Min), ApcΔ716, and Apc1638 mice (22–24). In the present study, we assessed hyperlipidemia in Apc1309 and Min mice by measuring serum levels of lipids and observed age-dependent increase of triglycerides, total cholesterol, and FFAs. As a possible cause, we found decreases of LPL mRNA levels in the liver and small intestine. We also investigated the effects of 100 or 200 ppm of pioglitazone and bezafibrate in the diet on both hyperlipidemia and intestinal polyposis in Apc1309 mice and demonstrated concomitant reduction in both. On the basis of these results for Apc gene-deficient mice, possible involvement of hyperlipidemia in intestinal polyp formation is proposed.

MATERIALS AND METHODS

Animals and Chemicals. Progeny of C57BL/6J/Apc1309 mice (Apc1309 mice), produced by a gene knockout method and bred by artificial insemination (21, 25), were obtained from CLEA Japan (Tokyo, Japan) at 5 weeks of age. Genotyping was performed using a three-oligonucleotide combination: 5’-TCAGGTTGAGCTCCATATCATCAGG-3’; 5’-CTCTGTGGGCAAG- ATCTTCCGTGAC-3’; and 5’-GCTAAGGCGAGTCTCCAGGCTG-3’. Genomic tail DNA was subjected to the PCR with the primers to amplify Apc alleles through 35 cycles of 94°C at 5 s, 62°C at 30 s, and 72°C at 30 s. Reaction products of 243 and 155 bp represent the ApcΔ1309 knockout and wild-type alleles, respectively. C57BL/6-ApcΔ1309 mice (Min mice) were purchased from The Jackson Laboratory (Bar Harbor, ME) and also genotyped according to the method described previously (26). Heterozygotes of these strains and wild-type (C57BL/6) mice were acclimated to laboratory condi-
tions for 1 week. Three to five mice were housed per plastic cage, with sterilized softwood chips as bedding, in a barrier-sustained animal room, air-conditioned at 24 ± 2°C and 55% humidity, on a 12-h light/dark cycle. Body weights and food consumption were measured weekly. The PPARγ ligand pioglitazone [1(±)-5-[4-[2-(5-ethyl-2-pyridyl)ethoxy]benzyl][thiazolidinedine-2,4-dione monohydrate] chloride] was kindly provided by Takeda Chemical Industries, Ltd. (Osaka, Japan), and the PPARα ligand bezafibrate [2-[4-(2-[4-chlorobenzenamido]ethyl)phenoxyl]-2-methylpropanoic acid] was purchased from Sigma Chemical (St. Louis, MO). These compounds were well mixed with powdered basal dietAIN-76A (CLEA Japan) at concentrations of 100 and 200 ppm.

**Experimental Design.** To assess change in serum lipid levels with aging, female Apc1309 and wild-type mice were randomly divided into four groups, each consisting of five animals, and fed a basal diet from 5 to 12 weeks of age. For comparison, female Min mice were randomly divided into three groups, each consisting of three or four mice, and fed a basal diet from 5 to 15 weeks of age. To investigate the effects of pioglitazone and bezafibrate on both hyperlipidemia and intestinal polyposis, 6–10 male Apc1309 mice were given (control), 100 or 200 ppm pioglitazone, or bezafibrate in the diet for 6 weeks, starting from 6 weeks of age. The doses were selected according to the results of a previous study, in which 100 ppm pioglitazone and bezafibrate in the diet starting from 6 weeks of age. The doses were selected according to the results of a previous study, in which 100 ppm pioglitazone and bezafibrate in the diet starting from 6 weeks of age. However, triglyceride levels were dramatically increased in Apc1309 mice thereafter (Fig. 1A), the average value at 12 weeks of age (618.2 ± 161.5 mg/dl) being almost 10 times higher than that at 6 weeks (72.0 ± 12.6 mg/dl). No such increase was observed in their wild-type counterparts. Total cholesterol in Apc1309 mice also significantly increased between 6 and 12 weeks of age (Fig. 1B), from 87.0 ± 3.2 mg/dl to 162.4 ± 33.0 mg/dl in contrast to the 70.2 ± 8.8 mg/dl to 79.6 ± 13.7 mg/dl found for the wild type. Significant changes in FFA levels also occurred with age (Fig. 1C). Serum lipid levels in male Apc1309 mice aged 12 weeks were almost the same as those in female Apc1309 mice at the same age (Fig. 2, A–C). Histopathologically, centrilobular-restricted steatosis was observed in the livers of all Apc1309 mice at 12 weeks of age, with numerous microvesicular fatty droplets in the cytoplasm of parenchymal cells (data not shown). Steatosis observed in Apc1309 mice was confirmed by staining frozen sections with Oil Red O. Wild-type mice exhibited no fatty change. The above observations indicate that Apc1309 mice develop hyperlipidemia as they age, the severity not differing between males and females.

In Min mice, triglyceride and FFA levels also increased dramatically with age (Fig. 1, D–F). Values for triglycerides in the serum of female Min mice at 8 and 15 weeks of age were 40.3 ± 6.2 and 377.3 ± 136.1 mg/dl, those for total cholesterol were 83.7 ± 6.3 and 107.8 ± 15.6 mg/dl, and those for FFAs were 10.0 ± 0.1 and 3.1 ± 0.4 mEq/liter, respectively. Histopathologically, centrilobular-restricted steatosis observed in the livers of all Apc1309 mice at 12 weeks of age, with numerous microvesicular fatty droplets in the cytoplasm of parenchymal cells (data not shown). Steatosis observed in Apc1309 mice was confirmed by staining frozen sections with Oil Red O. Wild-type mice exhibited no fatty change. The above observations indicate that Apc1309 mice develop hyperlipidemia as they age, the severity not differing between males and females.

**RESULTS**

**Elevation of Serum Lipid Levels in Apc Gene-deficient Mice.** Changes of serum lipid levels with ages were determined in female Apc1309 and wild-type mice. No significant differences were evident at 6 weeks of age. However, triglyceride levels were dramatically increased in Apc1309 mice thereafter (Fig. 1A), the average value at 12 weeks of age (618.2 ± 161.5 mg/dl) being almost 10 times higher than that at 6 weeks (72.0 ± 12.6 mg/dl). No such increase was observed in their wild-type counterparts. Total cholesterol in Apc1309 mice also significantly increased between 6 and 12 weeks of age (Fig. 1B), from 87.0 ± 3.2 mg/dl to 162.4 ± 33.0 mg/dl in contrast to the 70.2 ± 8.8 mg/dl to 79.6 ± 13.7 mg/dl found for the wild type. Significant changes in FFA levels also occurred with age (Fig. 1C). Serum lipid levels in male Apc1309 mice aged 12 weeks were almost the same as those in female Apc1309 mice at the same age (Fig. 2, A–C). Histopathologically, centrilobular-restricted steatosis was observed in the livers of all Apc1309 mice at 12 weeks of age, with numerous microvesicular fatty droplets in the cytoplasm of parenchymal cells (data not shown). Steatosis observed in Apc1309 mice was confirmed by staining frozen sections with Oil Red O. Wild-type mice exhibited no fatty change. The above observations indicate that Apc1309 mice develop hyperlipidemia as they age, the severity not differing between males and females.

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**Immunohistochemistry**. Expression and localization of PPARγ and PPARα in the small intestine were examined with rabbit polyclonal antibodies against each antigen using an avidin biotin complex method. Briefly, paraffin-embedded sections were deparaffinized and pretreated by heating in a microwave oven in 10 mM citrate buffer at pH 6.0 for 20 min. Nonspecific endogenous peroxidase activity was blocked by exposure to 0.5% hydrogen peroxide in methanol for 15 min, and masking was conducted with 5% normal goat serum in PBS containing 0.5% casein for 30 min. Incubation with anti-PPARγ (clone H-100; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-PPARα (clone H-98; Santa Cruz Biotechnology) was performed at 4°C, overnight. This step was followed by sequential incubation with biotin-labeled goat anti-rabbit IgG and avidin biotin complex reagents (Vector Laboratories, Burlingame, CA).

**Statistical Analysis.** The data for blood biochemistry and polyph formation are expressed as mean ± SE, and their statistical analysis was performed with Student’s t test. Ps < 0.05 were considered to be significant.

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Steatosis was apparent in the livers of the mice aged 15 weeks (data not shown).

Other serum biological parameters, such as glucose, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, and alkaline phosphatase, did not differ between groups of Apc-deficient mice, of either ApcΔf or Min strains, and wild-type mice at 6–15 weeks of age (data not shown).

Depression of Serum Lipid Levels in ApcΔf Mice by Pioglitazone and Bezafibrate. Administration of pioglitazone or bezafibrate did not affect food intake or behavior of ApcΔf mice. Final body weights in the 100 and 200 ppm pioglitazone-treated group were increased to 113–115% of those in the basal diet group, and those in bezafibrate-treated groups to 118–122%. Serum levels of triglycerides at 12 weeks of age decreased dose dependently, being reduced 44 and 50% by 100 and 200 ppm of pioglitazone, respectively (Fig. 2A). The levels of total cholesterol were also decreased by 15 and 28%, respectively, and administration of pioglitazone caused a 27% decrease in FFA levels in both 100 and 200 ppm groups, although these values were not statistically significant.

The size distribution of intestinal polyps in the basal diet and pioglitazone or bezafibrate-treated groups was investigated. Treatment with 100 and 200 ppm pioglitazone reduced the numbers of polyps measuring ≥1 and ≥0.5 mm in diameter, respectively (Fig. 3A). On the other hand, 100 and 200 ppm bezafibrate reduced the numbers of polyps, especially 0.5–1.5 mm in diameter (Fig. 3B).

Table 1  Data for intestinal polyps in ApcΔf mice treated with PPAR ligands

<table>
<thead>
<tr>
<th>Polyp location</th>
<th>Pioglitazone (ppm)</th>
<th>Bezafibrate (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (10)</td>
<td>100 (8)</td>
</tr>
<tr>
<td>Proximal small intestine</td>
<td>9.5 ± 1.1</td>
<td>5.8 ± 1.6</td>
</tr>
<tr>
<td>Middle small intestine</td>
<td>15.7 ± 1.1</td>
<td>11.4 ± 2.1</td>
</tr>
<tr>
<td>Distal small intestine</td>
<td>10.9 ± 1.2</td>
<td>7.0 ± 1.3</td>
</tr>
<tr>
<td>Colon</td>
<td>0.6 ± 0.2</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>Total</td>
<td>36.7 ± 2.7</td>
<td>24.6 ± 4.4</td>
</tr>
</tbody>
</table>

* Mice were fed the basal diet or a diet containing 100 or 200 ppm of PPAR ligand for 6 weeks.
* Numbers in parenthesis are the numbers of animals examined.
* Number of polyps per mouse.
* Data are means ± SE.
* Versus the basal diet group: P < 0.05.
* Versus the basal diet group: P < 0.01.
proteins and very low-density lipoproteins, respectively, but hepatic values for mRNAs were similar in all mouse strains, independent of the age. Administration of 100 and 200 ppm pioglitazone or bezafibrate raised the hepatic mRNA levels of LPL in Apc\(^{1309}\) mice (Fig. 5, A and B). A similar up-regulation was also evident for the small intestinal mRNA levels, although the degree of elevation was small (data not shown).

Fig. 3. Effects of pioglitazone (A) and bezafibrate (B) on size distribution of intestinal polyps in Apc\(^{1309}\) mice. Apc\(^{1309}\) mice were fed basal diet (closed box) or diet containing 100 ppm (open box) or 200 ppm (cross-hatched box) pioglitazone or bezafibrate for 6 weeks. Polyps were grouped at intervals of 0.5 mm according to their diameters. The number of polyps/mouse in each size class is expressed as the means; bars, SE.

Fig. 4. Decrease of LPL mRNA expression in the liver and small intestine of Apc\(^{1309}\) and Min mice. A, RT-PCR analysis of LPL mRNA expression in the liver of Apc\(^{1309}\) mice at 12 weeks of age and Min mice at 15 weeks of age. Wild-type mice for comparison were the same ages in each case. B, RT-PCR analysis of LPL mRNA expression in the normal mucosa (N) and polyp (P) of small intestine of Apc\(^{1309}\) and wild-type mice at 12 weeks of age. Data are representative of three separate experiments.

Fig. 5. Increase of LPL mRNA in the livers of Apc\(^{1309}\) mice attributable to pioglitazone and bezafibrate. RT-PCR analysis of LPL mRNA expression in the livers of Apc\(^{1309}\) mice at 12 weeks of age given diet containing pioglitazone (A) or bezafibrate (B) at doses of 0, 100, and 200 ppm for 6 weeks. Wild-type mice for comparison were the same ages in each case. Data are representative of three separate experiments.

PPAR\(\gamma\) and PPAR\(\alpha\) Expression in Small Intestinal Polyps. The presence of PPAR\(\gamma\) in the small intestines of female Apc\(^{1309}\) mice at 12 weeks of age was immunohistochemically confirmed in polyp epithelium and normal crypt epithelial cells. Diffuse staining of PPAR\(\gamma\) of epithelial cells of polyps and the bases of crypts was evident in the cytoplasm and nuclei (data not shown). Localization and staining intensity of PPAR\(\gamma\) in small intestinal epithelium of the wild-type mice was the same as in normal epithelium in Apc\(^{1309}\) mice. The expression pattern of PPAR\(\alpha\) was also similar to that for PPAR\(\gamma\). In the case of Min mice at 15 weeks of age, the localization and staining intensity of PPAR\(\gamma\) and PPAR\(\alpha\) in small intestinal epithelium were also the same as in Apc\(^{1309}\) mice. Control sections without primary antibodies showed no staining. Administration of pioglitazone or bezafibrate did not significantly alter the expression or localization of PPAR\(\gamma\) and PPAR\(\alpha\) in polyps and normal mucosa of Apc\(^{1309}\) mice when compared with the nontreated group.

DISCUSSION

The present study clearly demonstrated a hyperlipidemic state in two strains of FAP model mice. The levels of serum lipids, especially triglycerides, were thus dramatically increased with age in both Apc\(^{1309}\) and Min cases, with marked centrilobular-restricted steatosis observed in the livers. Moreover, LPL mRNA levels in the livers and small intestines of these mice were markedly lower than those of wild-type mice. Administration of the PPAR\(\gamma\) ligand, pioglitazone, or the PPAR\(\alpha\) ligand, bezafibrate, reduced both the serum level of triglycerides and intestinal polyp formation in the Apc\(^{1309}\) mice. The mRNA levels of LPL in the liver and small intestine were increased by pioglitazone or bezafibrate. It is therefore speculated that low levels of LPL mRNA expression may be associated with hyperlipidemia in Apc\(^{1309}\) and Min mice and involved in intestinal polyp development.

It has been reported that there are no accompanying increases in serum triglycerides and total cholesterol in rats with colon tumors induced by 1,2-dimethylhydrazine (36). We also confirmed no changes of serum lipid levels in C57BL/6 mice with colon tumors induced by AOM (data not shown). Therefore, tumor development itself may not cause hyperlipidemia. The deficiency in the Apc gene may be related not only to development of intestinal polyps but also to hyperlipidemia via decreased LPL gene expression. Inactivation of normal Apc function leads to accumulation of \(\beta\)-catenin and activation of the Wnt signaling pathway, in which the complex of \(\beta\)-catenin and the T-cell factor acts as a transcriptional factor. Thus far, c-myc, cyclin
D1, matrilysin, MDR1, gastrin, Id2, and PPARδ have been identified as target genes of Wnt signaling relevant to carcinogenesis (37–40). At present, the biological relationship between Apc deficiency and severe hyperlipidemic state is uncertain, but a report has been published that Wnt signaling maintains preadipocytes in an undifferentiated state through inhibition of the adipogenic transcription factors, CCAAT/enhancer binding protein α, and PPARγ (41). Moreover, transcriptional induction of the LPL gene has been reported to be mediated through binding of PPAR-retinoid X receptor heterodimers to the functional PPRE sequence in the LPL gene promoter (17). LPL catalyzes the rate-limiting step for clearance of triglycerides from the blood (17), and decrease of LPL mRNA levels results in elevation of serum lipid levels. Regarding lipid lowering drugs, fibrates predominantly affect liver LPL production through activation of PPARα (17). Moreover, the present study clearly showed that pioglitazone, as well as bezafibrate, raises the hepatic mRNA levels of LPL. It is therefore speculated that both PPARα and γ ligands might improve hyperlipidemia of Apc-deficient mice via increase of LPL mRNA levels in the liver.

On the other hand, different patterns of suppressive effects of polyp formation in Apc1309 mice were observed between PPAR α and γ ligands, i.e., numbers of polyps were reduced to a great extent by pioglitazone than by bezafibrate. Moreover, treatment with pioglitazone reduced the numbers of polyps of each size class, and bezafibrate only affected those with small sizes. These results suggested that pioglitazone might have additional mechanisms of suppression of intestinal polyp formation through PPARγ. Recently, Girnun et al. (42) reported that Pparg−/− mice exhibit greater β-catenin levels than Pparg+/+ mice, and a greater incidence of colon cancer was observed after treatment with AOM. Thus, PPARγ may act as a suppressor of the Wnt pathway, and the decreases of polyp numbers in Apc1309 mice by pioglitazone in the present study might be resulted from such suppression. Girnun et al. (42) also reported no difference in the number of colon tumors between Apc+/+1638N−/Pparg−/− and Apc+/+1638N−/Pparg+/+ mice at 65 weeks of age. However, the authors did not mention the serum lipid levels of these animals. Moreover, in contrast to Apc1309 and Min mice, the incidence and multiplicity of intestinal polyps in Apc+/+1638N− mice are very small (24). It is therefore speculated that the change of lipid metabolism in Apc-deficient mice may differ between strains, associated with the severity of polyp formation. A hyperlipidemic state could enhance the growth of intestinal polyps, and improvement of hyperlipidemia by treatment with PPAR ligands might thus reduce their size in Apc1309 mice. Furthermore, it has been reported that indomethacin, a nonsteroidal anti-inflammatory drug and cyclooxygenase inhibitor that suppresses intestinal polyp development in Min mice (43), can activate PPARα and γ in vitro (44). The relation between Apc deficiency and changes of lipid metabolism with age and the influence of hyperlipidemia on intestinal polyp development are now under detailed investigation in our laboratory.

It has been reported that polyp formation in the colon of the Min mice is enhanced by 2000 ppm or 150 mg/kg troglitazone, whereas in the small intestine is not affected (19, 20). In the present study, such promotion of colon polyp formation was not observed in Apc1309 mice treated with 100 and 200 ppm pioglitazone. Indeed, a clear suppressive effect on small intestinal polyp development was evident in these groups. Chemically induced ACF formation in the rat colon has also been shown to be suppressed by treatment with 100 ppm troglitazone and 100 ppm pioglitazone (18). Our preliminary study with a wide range of pioglitazone doses (50, 100, 200, 400, 800, and 1600 ppm in diet) in Apc1309 mice confirmed that small intestinal polyp development was suppressed at doses of 100 and 200 ppm and that the numbers of polyp in the colon and the small intestine were not increased up to 1600 ppm. It has been reported that low doses of PPARγ ligands are tumor promotive, whereas they are tumor suppressive at higher levels in breast cancer cells (45). Dose response effects of pioglitazone across a wide range on intestinal polyp formation in Min mice are also now under investigation in our laboratory.

In conclusion, the present study demonstrated that the PPAR ligands, pioglitazone and bezafibrate, have the potential to suppress both hyperlipidemia and polyp formation in Apc gene knockout mice. It is very important to now elucidate the mechanisms underlying the hypertriglyceridemia in FAP model mice and the roles of PPARγ and/or PPARα in intestinal polyp development.

REFERENCES


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