Chemopreventive Effect of Peroxisome Proliferator–Activated Receptor γ on Gastric Carcinogenesis in Mice

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Abstract
Peroxisome proliferator–activated receptor γ (PPARγ) is known to be expressed in several cancers, and the treatment of these cancer cells with PPARγ ligands often induces cell differentiation and apoptosis. Recently, the chemopreventive potential of PPARγ ligands on colon carcinogenesis was reported, although the effect of PPARγ on colon carcinogenesis and the mechanism of the effect remain controversial. In this study, we attempted to elucidate the role of PPARγ in gastric carcinogenesis and explored the possible use of a PPARγ ligand as a chemopreventive agent for gastric cancer. N-methyl-N-nitosourea (MNU, 240 ppm) was given in drinking water for 10 weeks to induce gastric cancer in PPARγ wild-type (+/+ ) and heterozygous-deficient (+/- ) mice, followed by treatment with PPARγ ligand [troglitazone, 0.15% (w/w) in powder food] or the vehicle alone for 42 weeks. At the end of the experiment, PPARγ (+/+ ) mice were more susceptible to MNU-induced gastric cancer than wild-type (+/+ ) mice (89.5%/55.5%), and troglitazone significantly reduced the incidence of gastric cancer in PPARγ (+/+ ) mice (treatment 55.5%/vehicle 9%) but not in PPARγ (+/- ) mice. The present study showed that (a) PPARγ suppresses gastric carcinogenesis, (b) the PPARγ ligand troglitazone is a potential chemopreventive agent for gastric carcinogenesis, and (c) troglitazone’s chemopreventive effect is dependent on PPARγ.

Introduction
Peroxisome proliferator–activated receptor gamma (PPARγ) is a member of a superfamily of nuclear hormone receptors (1). PPARγ heterodimerizes with retinoid X receptor to bind to the PPAR response element, leading to the transcription of downstream genes (2). PPARγ is known to be expressed in various organs, including adipose tissue (3), mammary glands (4), small intestine (5), lung (6), colon (5), and stomach (7), and is also up-regulated in various types of cancer cells. Several specific ligands have been identified, such as the thiazolidinediones (including pioglitazone, rosiglitazone, and troglitazone), 15-deoxy-prostaglandin-J2, and certain polyunsaturated fatty acids. PPARγ ligands have been reported to induce cell differentiation and apoptosis in several cancers (8–12), suggesting a potential application as anticancer agents. Furthermore, some reports recently suggested that PPARγ ligands can be used as chemopreventive agents for colon, breast, and tongue carcinogenesis (13–16). However, the effect of PPARγ ligands on colon cancer is controversial (17, 18). On the other hand, some recent studies have reported that the biological effect of PPARγ ligand is independent of PPARγ (19–23).

Whereas gastric cancer mortality has markedly declined around the world, it remains the second leading cause of cancer death worldwide (24, 25). Increasing interest has been shown in the chemoprevention of gastric cancer because of the low curable rate and the poor relative survival rate (26–29). Although the anticancer effect of PPARγ ligands has been reported in several gastric cancer cell lines (30–34), no information is available on the role of PPARγ in gastric carcinogenesis or whether PPARγ ligands actually inhibit gastric carcinogenesis.

To address the above questions, N-methyl-N-nitosourea (MNU) was used to induce gastric cancer in PPAR wild-type (+/+ ) and PPARγ heterozygous-deficient (+/- ) mice, followed by treatment with a PPARγ ligand, troglitazone, for 1 year. Our results clearly showed that PPARγ plays a protective role in gastric carcinogenesis and that the chemopreventative effect of troglitazone is dependent on PPARγ.

Materials and Methods

Animals. PPARγ knockout mice were generated as described previously (35, 36). Homozygous PPARγ knockout embryos (−/−) died because of placental dysfunction. We therefore used PPARγ heterozygous-deficient mice (+/−) in this study. To minimize the effect of the genetic background on carcinogenesis, one male and one female knockout mouse that had been generated by sister-brother mating for >10 generations were mated and all offspring were genotyped for the PPARγ gene. Then, all PPARγ heterozygous-deficient (−/+ ) offspring were mated again until the fourth generation mice were born. Both the wild-type and the heterozygous-deficient mice used in the present study were littermates. All mice were maintained in plastic cages with hardwood chip bedding in an air-conditioned room with a 12-hour light/12-hour dark cycle and given food (oriental CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) irradiated with 30 Gy of gamma rays and filtered tap water ad libitum.

Genetic typing. DNA was extracted from the ear of each mouse. Briefly, the ear was cut and the tissue was immersed into 400 mL of freshly prepared lysis buffer [80% saline sodium citrate, 2.5 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA (pH 8.0), 1% SDS, and 80 μg/mL proteinase K (Boehringer Mannheim GmbH, Indianapolis, IN)] and vortexed gently at 37°C overnight. Then 200 μL of phenol and 200 μL of chloroform were added to the lysate, and the mixture was rotated at room temperature for 1 hour, placed on ice for 5 minutes, and centrifuged at 15,000 rpm for 5 minutes. The aqueous phase was then transferred to a new tube. The above process was repeated a second time. Then, 400 μL of chloroform was added to the aqueous phase, and the solution was gently shaken by hand for 1 minute and centrifuged at

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15,000 rpm for 5 minutes. DNA was recovered by the addition of 400 µL isopropanol, decanted, and rinsed with 70% ethanol thrice. The DNA was dissolved in 100 µL of TE buffer. The concentration and purity of the DNA was examined with a spectrophotometer DU 60 (Beckman, Fullerton, CA). The extracted DNA was then subjected to a PCR.

PCR. PCR was done in a TaKaRa PCR Thermal Cycler 480 (TaKaRa Biomedicals, Shiga, Japan) in a total volume of 50 µL containing 100 ng DNA, PCR buffer, 4 µL deoxyribonucleotide triphosphate (TaKaRa Biomedicals), 0.5 unit Taq DNA polymerase (TaKaRa Biomedicals), 20 pmol of primer PPARY sense, 10 pmol of primer PPARY antisense, and 10 pmol of primer exon for 40 cycles of 30 seconds at 94°C for denaturation, 1 minute at 57°C for annealing, and 1 minute at 72°C for extension. The following primers were used: sense 5'-ctctatggcctagtctgc-3', antisense 5'-ggtattcttggagctt-3', and exon 5'-gccaccaaagaacggagccg-3'. The PCR product derived from the wild-type and knockout loci of PPARY were easily differentiated by agarose gel electrophoresis. The 400-bp band corresponds to the knockout allele, and the 300-bp band corresponds to the wild-type allele (data not shown).

The PPARY-specific ligand troglitazone was kindly provided by Sankyo Co., Ltd. (Tokyo, Japan). Troglitazone was mixed well in irradiated powder food containing 0.15% troglitazone, whereas groups 1, 2, 5, and 6 received distilled water. The mice in groups 3, 4, 7, and 8 were then given powder food for 42 weeks, until the end of the experiment. The carcinogen MNU 240 ppm in drinking water; the 4-µm-thick sections were stained with H&E and carefully examined. Well-differentiated adenocarcinomas were characterized by excessive glandular proliferation with pronounced structural and cellular atypia invading at least the submucosa, moderately differentiated adenocarcinomas by cellular atypia and atypical glandular structures, and carcinoma in situ by glandular proliferation with marked structural and cellular atypia within the gastric mucosa. Other macroscopically abnormal organs were also examined histologically.

Western blot analysis of peroxisome proliferator–activated receptor γ in mouse gastric mucosa. The expression of PPARY in the mouse stomach mucosa was examined by immunohistochemistry. The tissue sections were incubated at 4°C overnight with primary antibody for PPARY, and biotinylated polyclonal anti-rabbit immunoglobulin G/horseradish peroxidase (BD Biosciences, San Jose, CA) was used as the secondary antibody. Then visualization was done. The specificity of the binding was confirmed by omitting the primary antibody, and this staining was used as a negative control.

Statistical analysis. The incidences of gastric cancer were analyzed using Fisher's exact test. Survival curves were drawn using the Kaplan-Meier method and analyzed using the log-rank test. P < 0.05 was regarded statistically significant.

Results

Expression of peroxisome proliferator–activated receptor γ in mouse gastric mucosa. The expression of PPARY in PPARY wild-type (+/+ ) and heterozygous-deficient (+/− ) mice was examined immunohistochemically using a PPARY antibody. As shown in Fig. 2A, the expression of PPARY in wild-type (+/+) mice was significantly higher than that in heterozygous PPARY-deficient (+/− ) mice, whereas PPARY (+/− ) mice also exhibited a median expression of PPARY, compared with the negative control, because of the heterozygous deficiency of PPARY in these mice.

To confirm that PPARY is expressed at discernible levels in the mouse stomach, we also did Western blot analysis. Because PPARY is well known to be strongly expressed in the colon, the colon was used as a positive control. As shown in Fig. 2B, a considerable level of PPAR expression was detected in the stomach of the wild-type mice, although lower than that in the colon, and a weaker level of expression was detected in the heterozygous PPARY-deficient mice.

Loss of peroxisome proliferator–activated receptor γ promotes gastric carcinogenesis. At the end of the experiment, all surviving mice were killed and their stomachs and other organs were carefully examined. The tumor incidences are summarized in Table 1.

None of the mice in groups 1, 4, 5, and 8 developed cancer. The carcinoma MNU induced gastric carcinoma in 17 of 19 PPARY (+/− ) mice (group 2, 89.5%); one carcinoma in situ,
PPARγ and MNU-Induced Gastric Carcinogenesis

15 well-differentiated adenocarcinomas, one moderately differentiated adenocarcinoma and even one lymphoma in group 2 (+/- MNU, total of 19 mice). Gastric carcinoma was only induced in five of the nine wild-type mice that were examined (group 6, 55.5%; P < 0.05, compared with group 2; Table 1), all of which were well-differentiated gastric adenocarcinomas. These data suggest that PPARγ deficiency significantly sensitizes mice to MNU-induced gastric carcinogenesis.

Peroxisome proliferator-activated receptor γ activation by troglitazone prevents the development of gastric carcinogenesis. Among the wild-type mice, five of nine mice treated with MNU were found to carry gastric carcinoma (55.5%), but the administration of 0.15% troglitazone for 42 weeks significantly reduced the incidence of gastric carcinogenesis to 1 of 11 mice (9%). However, troglitazone did not inhibit carcinogenesis in the heterozygous PPARγ-deficient mice (MNU 89.5%, MNU + Tro 80%; Table 1). Therefore, the preventative effect of troglitazone was considered dependent on PPARγ.

No appreciable histologic difference was observed in gastric carcinomas between wild-type and heterozygous peroxisome proliferator-activated receptor γ-deficient mice. No significant macroscopic differences in the gastric tumors were observed between the wild-type and PPARγ-deficient mice. The microscopic morphology of the tumors was also carefully examined. Representative images of the macroscopic appearance of stomach adenocarcinoma (Fig. 3A) and the microscopic appearance of normal stomach mucosa (Fig. 3B), well-differentiated adenocarcinoma (Fig. 3C), and moderately differentiated adenocarcinoma (Fig. 3D), carcinoma in situ (Fig. 3E), and lymphoma in stomach (Fig. 3F) are shown. The gastric adenocarcinomas were mainly located in the pyloric mucosa and occasionally at the fundopyloric border. No appreciable histologic difference in the gastric carcinomas was observed between the wild-type and heterozygous PPARγ-deficient mice.

Mice died after the 37th week because of carcinogen-induced gastric carcinoma. The survival curves for all eight mice groups are shown in Fig. 4. Before the end of the experiment (52nd week), 10 of 19 mice in group 2, 8 of 15 mice in group 3, 1 of 9 mice in group 6, and 1 of 11 mice in group 7 died. These data suggested that the increased gastric carcinoma burden reduced the survival time of PPARγ (+/-) mice, compared with that of wild-type mice (P < 0.01), but there was no difference between the control group and the troglitazone-treated group, either wild-type or PPARγ (+/-) mice. Because of the reported rare but severe hepatotoxicity of troglitazone, we examined the liver of mice treated with troglitazone. Neither degeneration nor necrosis was observed. Focal liver cell hyperplasia was found in one mouse in the group 3, but no dysplasia or cancer was observed. We did not find any abnormality in other organs.

Table 1. Incidence of stomach lesions in eight groups of mice at experiment week 52

<table>
<thead>
<tr>
<th>Group</th>
<th>PPARγ</th>
<th>MNU</th>
<th>Tro</th>
<th>No. mice</th>
<th>Effective no. mice</th>
<th>Total incidence of carcinoma (%)</th>
<th>Well-differentiated</th>
<th>Moderately differentiated</th>
<th>Carcinoma in situ</th>
<th>Lymphoma</th>
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<td>0</td>
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<tr>
<td>2</td>
<td>+/-</td>
<td>–</td>
<td>+</td>
<td>23</td>
<td>19</td>
<td>17 (89.5)*</td>
<td>15 (78.9)</td>
<td>1 (5.3)</td>
<td>1 (5.3)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>3</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>19</td>
<td>15</td>
<td>12 (80)</td>
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<td>0</td>
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<td>0</td>
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<tr>
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<td>+</td>
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<td>–</td>
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<td>7</td>
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<td>6</td>
<td>+/-</td>
<td>+</td>
<td>–</td>
<td>11</td>
<td>9</td>
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*P < 0.01, group 6.
†P < 0.01, group 7.
Peroxisome proliferator–activated receptor γ expression was suppressed in cancerous gastric mucosa. The above observations suggest that PPARγ might exert a tumor suppressor activity in response to endogenous ligand(s). If this is the case, the gastric carcinomas that were induced might have lost their expression of PPARγ because of inhibitory selective pressure from either endogenous or exogenous ligands. To address this question, we examined the expression of PPARγ in cancerous and normal gastric mucosa from the same mouse using immunohistochemistry. The results showed that the expression of PPARγ in the malignant mucosa was much weaker than in the normal mucosa in both wild-type and heterozygous PPARγ-deficient mice, as shown in Fig. 5A, and the expression of PPARγ was also lower in the cancerous mucosa of the wild-type mouse treated with troglitazone that developed gastric carcinoma, although a stronger PPARγ expression was observed in the normal mucosa of mice treated with troglitazone. This result was confirmed by Western blot analysis using the protein extracted from either the normal or the cancerous gastric mucosa from wild-type mice (Fig. 5B).

Discussion

Recently, the potential of PPARγ as a target for the prevention and treatment of cancer has been widely explored (38). However, the therapeutic potential of PPARγ ligands has been questioned, based on the results of experiments using animal models for colon cancer, in which the PPARγ ligands increased the development of colon tumors (17). This contradictory result was supplemented by a recent report using transgenic mice that express a constitutive active form of PPARγ in mammary glands showing that PPARγ signaling accelerated tumor development in mammary glands (39). The actual role of PPARγ in cancer has been complicated by recent findings that PPARγ ligands affect cancer cells independent of PPARγ (21, 40–42). To date, no information on the role of PPARγ in gastric carcinogenesis is available.

The results of this study provide clear evidence of the critical importance of PPARγ in gastric carcinogenesis. The loss of one allele of PPARγ significantly enhanced carcinogen-induced gastric carcinogenesis and decreased the survival rate, compared with the wild-type (+/+) littermates. Our results are in good agreement with the report by Nicol et al. that PPARγ haploinsufficiency increased the susceptibility to carcinogen-induced breast carcinogenesis, suggesting that PPARγ acts as a tumor suppressor of skin, ovarian, and breast cancers (43). However, our results are inconsistent with those of one other study that suggested PPARγ acts as a tumor promoter in breast carcinogenesis, instead of a tumor suppressor (39). In that study, the loss of one allele of PPARγ did not influence breast tumorigenesis. This result cannot be easily explained. However, the histologic and pathologic differences between breast
and stomach cancer, the method of inducing carcinogenesis (mouse mammary tumor virus/PyV transgenic mice were used to evaluate tumorigenesis in the other study, whereas the carcinogen MN1 in drinking water was used to induce carcinogenesis in the present study), and the difference in the genetic backgrounds of the mice used in the two studies [the (C57BL/6 × DBA/2)F1 (B6D2F1) mice were used in that study and the B6 × CBA × ICR mice in the present study] might be responsible for these inconsistencies, although further investigation is certainly needed.

The reduction in PPARγ expression observed among MNU-induced gastric adenocarcinomas from either PPARγ (+/-) or wild-type mice agreed well with the findings of several other reports showing a reduction in PPARγ protein expression in breast and colon cancers, where expression was highest in normal tissue and decreased from benign to malignant states of disease (44–46). Recently, Badawi et al. reported that PPARγ mRNA and protein levels were lower in MNU-induced rat mammary tumors than in normal tissues (47). Our present study showed the suppressed expression of PPARγ in MNU-induced carcinomas, showing a consistent pattern of PPARγ expression. These observations support the hypothesis that PPARγ can exert a tumor suppressing activity.

Furthermore, our results unambiguously showed the chemopreventive potential of a PPARγ ligand, troglitazone, in gastric carcinogenesis. The administration of troglitazone significantly suppressed the formation of MNU-induced gastric carcinoma in wild-type mice. No significant differences in the macroscopic and histologic features of the gastric adenocarcinomas were observed between the wild-type and PPARγ-deficient mice treated with or without troglitazone. Importantly, troglitazone suppressed gastric carcinogenesis without affecting the tumor pathology. In addition, troglitazone’s preventive effect was only observed in wild-type mice but not in heterozygous PPARγ-deficient mice, and the reduction in PPARγ expression in the transformed mucosa clearly indicated that this effect was dependent of PPARγ. This result addresses the controversy regarding the dependence of troglitazone on PPARγ, at least in gastric carcinogenesis. However, in the present study, within 52 weeks, troglitazone did not alter the mortality rate. Only one mouse died in the wild-type mice group, either treated with or without troglitazone. It is necessary to extend the experimental period to evaluate troglitazone’s effect on the mortality rate. Because troglitazone’s preventive effect on gastric cancer seems dependent of PPARγ, as observed in the present study, the preventive effect of other ligands, such as pioglitazone, rosiglitazone and 15-prostaglandin-J2, on carcinogenesis should be investigated in the future. Recently, the preventive effect of PPARγ against acute gastric mucosal lesions associated with ischemia-reperfusion was reported (48). In that study, PPARγ ligands showed protection against acute gastric mucosal lesions formation induced by ischemia-reperfusion in mice in a dose-dependent manner, and the acute gastric mucosal lesions in PPARγ (+/-) mice was more severe than in wild-type mice. In addition, the inhibition of the up-regulation of tumor necrosis factor-α, intercellular adhesion molecule-1, inducible nitric oxide synthase, apoptosis, and nitrotyrosine formation in the stomach may be responsible for the preventive effect of PPARγ. This may provide us the speculation that the preventive effect of PPARγ against gastric carcinogenesis may be through inhibiting the nuclear factor-κB-mediated transcription.

Our present results that PPARγ deficiency sensitizes mice to carcinogen-induced gastric carcinogenesis may provide a way of identifying certain populations susceptible to gastric cancer. In addition, the significant cancer-preventive effect of troglitazone has very important clinical implications. Provided that certain individuals at a higher risk of gastric cancer can be identified, these individuals might actually benefit from the use of PPARγ ligands as chemopreventive agents for gastric cancer.

Taken as a whole, the present study is the first report to show that (a) PPARγ (+/-) mice have an increased susceptibility to MNU-induced carcinogenesis, suggesting that PPARγ may function as a tumor suppressor; (b) the PPARγ ligand troglitazone is a potential chemopreventive agent for gastric cancer; and (c) troglitazone’s chemopreventive effect is dependent on PPARγ.

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