RESEARCH ARTICLE

Suppressive Effect of Pioglitazone, a PPAR Gamma Ligand, on Azoxymethane-induced Colon Aberrant Crypt Foci in KK-A^y Mice

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Abstract

Obesity is an established risk factor for colorectal cancer. Pioglitazone is a peroxisome proliferator-activated receptorγ (PPARγ) agonist that induces differentiation in adipocytes and induces growth arrest and/or apoptosis *in vitro* in several cancer cell lines. In the present study, we investigated the effect of pioglitazone on the development of azoxymethane-induced colon aberrant crypt foci (ACF) in KK-A^y obesity and diabetes model mice, and tried to clarify mechanisms by which the PPARγ ligand inhibits ACF development. Administration of 800 ppm pioglitazone reduced the number of colon ACF / mouse to 30% of those in untreated mice and improved hypertrophic changes of adipocytes in KK-A^y mice with significant reduction of serum triglyceride and insulin levels. Moreover, mRNA levels of adipocytokines, such as leptin, monocyte chemoattractant protein-1 and plasminogen activator inhibitor-1, in the visceral fat were decreased. PCNA immunohistochemistry revealed that pioglitazone treatment suppressed cell proliferation in the colorectal epithelium with elevation of p27 and p53 gene expression. These results suggest that pioglitazone prevented obesity-associated colon carcinogenesis through improvement of dysregulated adipocytokine levels and high serum levels of triglyceride and insulin, and increase of p27 and p53 mRNA levels in the colorectal mucosa. These data indicate that pioglitazone warrants attention as a potential chemopreventive agent against obesity-associated colorectal cancer.

Key words: Pioglitazone - obesity - PPAR gamma - aberrant crypt foci

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Introduction

Colorectal cancer is one of the common cancers in developed countries including Japan. Many epidemiological studies have suggested colorectal cancer correlates with obesity, a high-fat diet and hyperlipidemia, especially, hypertriglyceridemia and high levels of low-density lipoprotein cholesterol (Le Marchand et al., 1997; Bruce et al., 2000). Assumed mechanisms underlying obesity-associated cancer development could involve insulin resistance, chronic inflammation and dyslipidemia caused by dysregulation of adipocytokine production. Among adipocytokines, increased levels of leptin, plasminogen activator inhibitor-1 (PAI-1), and decreased levels of adiponectin are demonstrated to play an important role in colorectal carcinogenesis (van Kruijsdijk et al., 2009).

Recently, we have reported that KK-A^y mice, carrying the Agouti yellow gene (A^y) and resultant hyperphagia (Nakamura et al., 1967), are highly susceptible to azoxymethane (AOM)-induced colorectal carcinogenesis (Teraoka et al., 2011). The KK-A^y mice exhibited severe abdominal obesity, hypertriglyceridemia and hyperinsulinemia. Moreover, serum pro-inflammatory adipocytokines such as interleukin-6 (IL-6), leptin and Pai-1 in KK-A^y mice were elevated and adiponectin was decreased compared to those in lean C57BL/6J mice. Among them, serum leptin levels were the highest in KK-A^y mice. Those features of KK-Ay mice could explain their high susceptibility to AOM-induced colorectal carcinogenesis, and suggests they could be useful to evaluate chemopreventive agents against obesity-associated colorectal cancer.

Peroxisome proliferator-activated receptorγ (PPARγ) is a key nuclear hormone receptor of lipid metabolisms and regulates several gene transcriptions associated with

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differentiation, growth arrest and apoptosis (Fisher et al., 1998; Sporn et al., 2000). PPARγ directly activates lipoprotein lipase (LPL) promoter activity, and induces LPL, which catabolizes triglycerides to monoglycerides (Schoonjans et al., 1996). Activation of PPARγ also induces terminal differentiation of adipocytes linking to downsizing of hypertrophic adipotissue. Meanwhile, PPARγ induces growth arrest and apoptosis in several cancer cell lines, including colon, esophageal squamous, gastric and pancreatic cancer cells (Takahashi et al., 1999; Shimada et al., 2002; Rumi et al., 2002; Itami et al., 2001). Pioglitazone is a selective PPARγ agonist that improves hyperlipidemia and hyperglycemia in obese diabetic animals and humans (Sohda et al., 1990; Ikeda et al., 1990; Sakamoto et al., 2000). Although side effects, such as weight gain, peripheral edema, precipitation of chronic heart failure and an increase in bone fractures limit widespread use of pioglitazone, pioglitazone is a useful antidiabetic drug, which is well tolerated in the majority of patients (Shah et al., 2010). Previously, we have reported that pioglitazone induced LPL and suppressed concurrently both hyperlipidemia and intestinal polyp formation in *Apc*-deficient Min mice, a model mouse for familial adenomatous polyposis (Niho et al., 2003). Thus, pioglitazone may be a potential chemopreventive agent against colorectal carcinogenesis. Furthermore, pioglitazone may be a more useful chemopreventive agent against obesity-associated cancers, such as mammary cancer (Bojková et al., 2010).

In the present study, we investigated the effects of pioglitazone on the development of AOM-induced aberrant crypt foci (ACF) in obese KK-A^y mice. The novelty of this study is investigating the effect of pioglitazone in obese mice with retention of leptin and leptin receptor genes, in which we are able to examine actions of several molecules, such as adipocytokine, triglyceride and insulin with intact leptin signaling. The results demonstrated that pioglitazone prevented obesity-associated colorectal carcinogenesis through improving dysregulated levels of adipocytokines, especially leptin, insulin and lipids. Furthermore, another mechanism underlying the suppressive effect of pioglitazone is discussed with reference to induction of cell cycle-related genes.

Materials and Methods

Animals and chemicals

Female 5-week-old KK-A^y/TaJcl (KK-A^y) and C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan), and acclimated to laboratory conditions for 1 week. Five mice were housed per plastic cage with sterilized softwood chips as bedding in a barrier-sustained animal room at 24 ± 2°C and 55% humidity on a 12 hr light/dark cycle and fed AIN-76A powdered basal diet (CLEA Japan) and water. The animals in each cage were all in the same treatment group. The pioglitazone, {(±)-5-[4-[2-(5-ethyl-2-pyridyl)ethoxy] benzyl]thiazolidine-2,4-dione monohydrochloride}, was kindly provided by Takeda Chemical Industries, Ltd. (Osaka, Japan).

Experimental protocol for KK-A^y mice treated with azoxymethane and pioglitazone

For the induction of ACF by AOM (Nard Institute, Ltd., Amagasaki, Japan), 6-week-old female KK-A^y (n=10) were given intraperitoneal injections of AOM (200 μg/mouse) in 0.9% NaCl saline once a week for 3 weeks. Five mice were also injected with saline as a control group. At the same time of first intraperitoneal injections, pioglitazone was administrated at concentrations of 400 or 800 ppm in basal diet. The dosage of pioglitazone was determined by our previous experiment (Niho et al., 2003). Food and water were available ad libitum. The animals were observed daily for clinical signs and mortality. Body weights and food and water consumption were measured weekly. All the mice were anesthetized with ether and sacrificed at the age of 13 weeks, the organs, including intestinal tract, heart, kidneys, liver, lungs, spleen and visceral fat, were excised and were also observed macroscopically and blood samples from the caudal vena cava were collected. A part of visceral adipose tissue and liver tissue of KK-A^y mice with and without AOM treatment, and colon mucosa of KK-A^y mice without AOM treatment were rapidly deep-frozen in liquid nitrogen and stored at -80°C for further experiments. The experiments were performed according to the "Guidelines for Animal Experiments in the National Cancer Center" and were approved by the Institutional Ethics Review Committee for Animal Experimentation in the National Cancer Center.

Assessment of AOM-induced colorectal ACF

The intestinal tract was removed, the colorectum opened longitudinally and fixed flat between sheets of filter paper in 10% buffered formalin. After dividing the colorectum into the proximal segment and rectum (1.5 cm in length), halves of the remainder were divided into the middle and distal segment. These were stained with 0.2% methylene blue (Merck, Darmstadt, Germany) and the mucosal surface was assessed for ACF with a stereoscopic microscope, as previously reported (Bird et al., 1987).

Analysis of visceral adiposity

The images of visceral and subcutaneous fat were obtained by a cone-beam micro-CT scanner (eXplore Locus, General Electric Healthcare, Ontario, Canada) scanning from the first lumbar vertebra to the pubic bone. The volumes of the fat were analyzed by MicroView software (General Electric Healthcare).

Real-time polymerase chain reaction analysis

Total RNA was isolated from tissues by using Isogen (Nippon Gene, Tokyo, Japan), and treated with DNase I (Invitrogen, Carlsbad, CA, USA). One-μg RNA in a

Table 1. Development of Colorectal ACF in KK-A^y Mice Treated with AOM and Pioglitazone

Pioglitazone	No. of mice	No. of ACF / colorectum					Mean no.
(ppm)	with ACF	Proximal	Middle	Distal	Rectum	Total	of ACs / focus
0	11 / 11	5.9 ± 3.3	19.1 ± 8.2	18.6 ± 5.2	6.9 ± 1.9	50.5 ± 13.9	1.6 ± 0.2
400	10 / 10	1.6 ± 2.6 **	6.5 ± 3.6 **	22.2 ± 9.3	9.7 ± 5.7	40.0 ± 13.4	1.4 ± 0.2
800	10 / 10	0.1 ± 0.3 **	3.8 ± 2.3 **	19.6 ± 6.2	8.4 ± 2.8	31.9 ± 6.6 *	1.5 ± 0.2

Data are means ± SD. *p<0.05, **p<0.01 vs 0 ppm

final volume of 20 μL was used for synthesis of cDNA using an Omniscript® RT Kit (Qiagen, Hilden, Germany) with an oligo (dT) primer. Real-time PCR was carried out using a DNA Engine OpticonTM 2 (MJ Japan Ltd., Tokyo, Japan) with SYBR Green Real-time PCR Master Mix (Toyobo Co., Osaka, Japan). Primers for mouse adiponectin (5'-AGGATGCTACTGTTGCAAGCTCTC, 3'- CAGTCAGTTGGTATCATGGTAGAG), GAPDH (5'- TTGTCTCCTGCGACTTCA, 3'- CACCACCCTGTTGCTGTA), IL-6 (5'- ACAACCACGGCCTTCCCTACTT, 3'- CACGATTTCCCAGAGAACATGTG), leptin (5'- CCAAAACCCTCATCAAGACC, 3'- GTCCAACTGTTGAAGAATGTCCC), LPL (5'- GGATCCGTGGCCGCAGCAGACGCAGGA, 3'- GAATTCCATCCAGTTGATGAATCTGGCCAC), monocyte chemoattractant protein (MCP-1) (5'- CCACTCACCTGCTGCTACTCAT, 3'- TGGTGATCCTCTTGTAGCTCTCC), Ob-Rbl (5' Primer- CCATCTTTTATATGATCTGCCTGAAGT, 3' Primer- TGCATTGGACAGTCTGAAAGCT), Pai-1 (5'- ACAGCCTTTGTCATCTCAGCC, 3'- AGGGTTGCACTAAACATGTCAG), tumor necrosis factor-a(TNF-a) (5'- TGTGCTCAGAGCTTTCAACAAC, 3'- GCCCATTTGAGTCCTTGATG), p27 (5'- TCTCAGGCAAACTCTGAGGA, 3'- CTTCCTCATCCCTGGACACT), p53 (5'- CCCCAGGATGTTGAGGAGTTT, 3'- TTGAGAAGGGACAAAAGATGACA) were employed (Teraoka et al., 2011; Niho et al., 2005; Turmelle et al., 2006; Xiao et al., 2009). To assess the specificity of each primer set, amplicons generated from the PCR reaction were analyzed for melting curves.

Measurement for serum lipids, adipocytokine and insulin

Serum levels of triglycerides and total cholesterol were measured as reported (Niho et al., 2003). Serum levels of adiponectin, interleukin-1β (IL-1β), IL-6, MCP-1, leptin and insulin were measured using mouse adiponectin immunoassay (R&D systems, Inc., Minneapolis, MN, USA), mouse procarta® cytokine assay (Affymetrix, Inc., Santa Clara, CA, USA), a mouse leptin enzyme-linked immunosorbent assay kit (B-Bridge International, Inc., Cupertino, CA, USA) and a mouse insulin kit (Millipore Corp., Billerica, MA, USA).

Immunohistochemical analysis

The colon (segment of middle and distal) after analysis of colon ACF formation and the liver and the visceral fat were sliced and processed to sections stained with hematoxylin and eosin (H&E). Sections of middle and distal colon were also stained immunohistochemically with antibodies against proliferation cell nuclear antigen (PCNA; DAKO, Carpinteria, CA, USA) used at 200 x dilution. The number of PCNA positive cells was measured in a crypt from three different arbitrarily selected points in colon mucosa (n=5). The extent of enlargement of adipocytes was evaluated by quantification of the number of adipocyte nuclei observed in the field (x 200) of fat tissue in KK-A^y mice.

Statistical analysis

The significance of difference in the number of AOM-induced colorectal ACF, serum lipid levels and serum cytokine levels was analyzed using Dunnett's multiple comparison test and other statistical analyses were performed with Student's t-test. Differences were considered to be statistically significant at $p<0.05$.

Results

Suppression of the numbers of AOM-induced colorectal ACF in KK-Ay mice by pioglitazone

To determine the effect of pioglitazone on colorectal ACF development in obese KK-A^y mice, KK-A^y mice were treated with AOM and pioglitazone. Administration of pioglitazone did not significantly effect food intake, behavior or body weight changes during the experiment periods. Final body weights in 13-week-old female KK-A^y mice untreated, treated with 400 ppm pioglitazone and 800 ppm pioglitazone were 45.7 ± 3.1 (mean ± SD), 40.0 ± 3.4 and 42.0 ± 3.5 g, respectively.

Table 1 shows data for the numbers and distribution of colorectal ACF in KK-A^y mice with or without pioglitazone. All KK-Ay mice treated with AOM developed ACF in the colorectum at 13 weeks. The total numbers of ACF in the groups treated with pioglitazone at 400 and 800 ppm doses were reduced to 79 and 63 % ($p<0.05$) of the control value, respectively. Of note, the number of ACF in the proximal and middle parts of the colon in the mice fed diet containing 400 and 800 ppm pioglitazone were reduced significantly ($p<0.01$). There were no significant differences in the mean numbers of ACs per focus among each group.

Improvement of fatty change in the liver and hypertrophy of adipocytes in the visceral fat tissue by pioglitazone

To clarify the effects of AOM and pioglitazone on other tissue, histopathological examination were

Table 2. Amount of Fat Tissue in KK-*Ay* Mice Treated with AOM and Pioglitazone

Pioglitazone (ppm)	Visceral fat (g)	Subcutaneous fat (g)	Total (g)
0	6.2 ± 0.9	2.0 ± 0.4	8.2 ± 1.2
800	4.2 ± 0.4 **	1.6 ± 0.1 *	5.9 ± 0.5 **

Data are means ± SD. *p<0.05, **p<0.01 vs 0 ppm; Amount of fat tissue was analyzed by micro-CT

Table 3. Levels of Serum Lipids and Insulin in KK-*Ay* Mice Treated with AOM and Pioglitazone

Pioglitazone (ppm)	Triglycerides (mg / dL)	Total cholesterol (mg / dL)	Insulin (ng / mL)
0	502.0 ± 128.4	103.8 ± 19.3	39.5 ± 2.8
400	262.8 ± 81.2**	89.7 ± 10.9	3.5 ± 1.6**
800	254.3 ± 62.3 **	97.7 ± 16.8	4.4 ± 3.5 **

Data are means ± SD. **p<0.01 vs 0 ppm

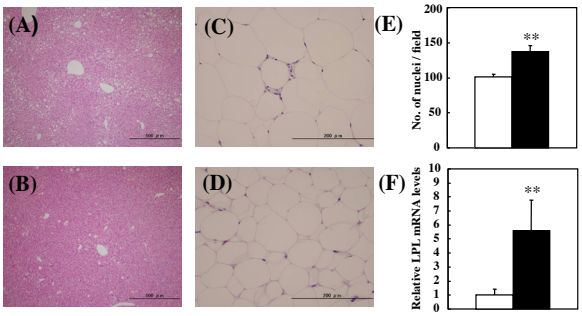

Figure 1. Fatty Change in the Liver and Size of Adipocytes in the Visceral Fat Tissue. (A, B) Representative histopathological sections (H&E) of the liver in KK-*Ay* mice and the liver of the mice treated with 800 ppm pioglitazone are shown, respectively. (C, D) Representative histopathological sections (H&E) of the visceral fat in KK-*Ay* mice and the mice treated with 800 ppm pioglitazone, respectively. (E) Numbers of nuclei of visceral fat cells in a microscopical field were counted and are shown. (F) Hepatic LPL mRNA expression levels were examined by real-time PCR analysis. GAPDH mRNA was used to normalize the data. Values were set at 1.0 in untreated control Data are means ± SE (n=5). **, p<0.01.

performed on the liver and visceral fat tissue. The weights of liver at the end of experiments in KK-*Ay* mice treated with AOM and 0, 400 and 800 ppm pioglitazone treated group were 1.7 ± 0.1 (mean ± SD), 1.7 ± 0.2 and 1.9 ± 0.2 g, respectively. As shown in Figure 1A, fatty change of the liver was observed in KK-*Ay* mice by histopathological examination, and was clearly improved by administration of 800 ppm pioglitazone (Figure 1B).

The volume of visceral and subcutaneous fat was measured by a micro-CT, and these amounts are summarized in Table 2. Treatment with 800 ppm pioglitazone in KK-*Ay* mice significantly decreased the amount of visceral fat (p<0.01), subcutaneous fat (p<0.05) and total fat (p<0.01) compared with those of the untreated control. The amounts of mesenteric fat tissue in KK-*Ay* mice untreated, treated with 400 ppm and 800 ppm pioglitazone were 1.5 ± 0.4 (mean ± SD), 0.7 ± 0.2 (p<0.01 vs 0 ppm) and 0.7 ± 0.3 g (p<0.01 vs 0 ppm), respectively. Histopathological examination of visceral adipose tissue clearly showed that the size of adipocytes in visceral adipose tissue in KK-*Ay* mice treated with 800 ppm pioglitazone was smaller than that in untreated control mice (Figure 1C and 1D). Moreover, the number of adipocyte nuclei observed in one field of visceral fat tissue under the microscope (x200) in KK-*Ay* mice was 101.0 ± 10.0, and treatment with 800 ppm pioglitazone significantly increased the number to 137.0 ± 20.0 (p<0.01) (Figure 1E).

Improvement of the levels of lipids, insulin and adipocytokines in serum of KK-Ay mice treated with pioglitazone

To evaluate the effects of pioglitazone on the size reduction of adipocytes in the visceral fat tissue, the levels of serum lipids, insulin and adipocytokines were measured, and are summarized in Tables 3 and 4. The average serum levels of triglycerides and insulin, but not total cholesterol of KK-*Ay* mice treated with AOM and 400 ppm or 800 ppm pioglitazone were significantly decreased compared with those of KK-*Ay* mice treated AOM alone (Table 3). Thus, we investigated the effects of pioglitazone on hepatic mRNA levels of LPL,

Table 4. Levels of Serum Adipocytokines in KK-*Ay* Mice Treated with AOM and Pioglitazone

Pioglitazone (ppm)	Adiponectin (mg / mL)	IL-1β (pg / mL)	IL-6 (pg / mL)	MCP-1 (pg / mL)	Leptin (ng / mL)
0	12.7 ± 1.5	54.1 ± 17.6	26.4 ± 20.4	155.1 ± 37.2	140 ± 31.7
400	14.5 ± 0.9	36.6 ± 6.80*	12.2 ± 2.60	138.6 ± 26.9	45.9 ± 27.2**
800	27.9 ± 2.0 **	51.4 ± 10.7	35.0 ± 20.8	99.3 ± 36.1**	46.5 ± 21.7 **

Data are means ± SD. **p<0.01 vs 0 ppm, . *p<0.05 vs 0 ppm

Table 5. PCNA Immunostaining in Middle or Distal Colon of KK-*Ay* Mice Treated with AOM and 800 ppm Pioglitazone

Pioglitazone (ppm)	Cells / crypt		PCNA positive cell / crypt		% of PCNA positive cells / total cells crypt	
	Middle	Distal	Middle	Distal	Middle	Distal
0	42.0 ± 4.2	39.6 ± 3.3	17.0 ± 4.7	12.2 ± 2.7	41.2 ± 11.4	30.6 ± 7.0
800	38.6 ± 3.5	34.2 ± 3.9	12.9 ± 2.7	12.1 ± 2.7	33.4 ± 5.8	35.8 ± 8.8

Data are means ± SD

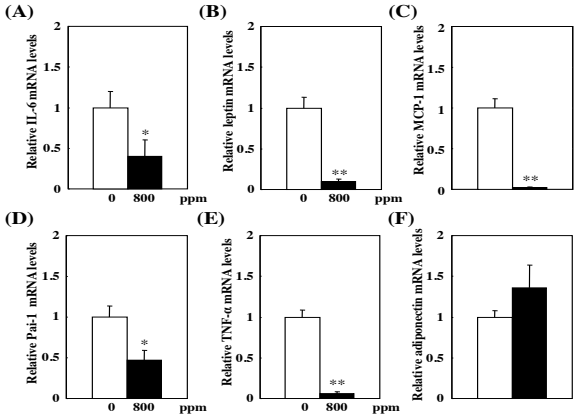

Figure 2. Relative Expression Levels of Adipocytokine mRNA in Visceral Fat Tissue of KK-Ay Mice. Real-time PCR analysis was performed to obtain IL-6 (A), leptin (B), MCP-1 (C), Pai-1 (D), TNF-α (E) and adiponectin (F) mRNA expression levels. GAPDH mRNA was used to normalize the data. White, untreated control group. Black, 800 ppm pioglitazone treated group. Values were set at 1.0 in the untreated control. Data are means ± SE (n=5). *, p<0.05, **, p<0.01.

which catabolizes triglycerides to monoglycerides. Administration of 800 ppm pioglitazone increased 5.6-fold the hepatic LPL mRNA levels compared with those of untreated control levels (Figure 1F). The serum adiponectin level was also significantly increased and serum levels of leptin and MCP-1 were significantly decreased in KK-Ay mice treated with 800 ppm pioglitazone compared with untreated KK-*Ay* mice (p<0.01). Significant differences were also obtained in serum levels of IL-1β and leptin in KK-*Ay* mice treated with 400 ppm pioglitazone (Table 4).

Improvement of the levels of adipocytokines in visceral fat tissue of KK-Ay mice treated with pioglitazone

Data for the mRNA expression levels of adipocytokines in visceral fat tissue are shown in Figure 2. The mRNA expression levels of IL-6, leptin, MCP-1, Pai-1 and TNF-α in KK-*Ay* mice treated with AOM and 800 ppm pioglitazone were significantly decreased compared with those of KK-Ay mice treated with AOM alone. On the other hand, treatment of 800 ppm pioglitazone had a tendency to up-regulate the mRNA expression of adiponectin compared with untreated KK-*Ay* mice.

Validation of colon epithelial cell proliferation in KK-Ay mice treated with pioglitazone and/or AOM

To investigate the effect of pioglitazone treatment on epithelial cell proliferation of colon mucosa in KK-*Ay* mice, the amount of cells in S phase and expression of cell cycle-related gene (p27 and p53) were examined. PCNA immunohistochemical staining revealed that administration of 800 ppm pioglitazone had a tendency to suppress cell proliferation in the colon mucosa in KK-*Ay* mice. As shown in Table 5, the total cells per crypt in middle colon mucosa of KK-*Ay* mice and the mice treated with 800 ppm pioglitazone were 41.2 ± 11.4 and 33.4 ± 5.8, and PCNA positive cells in those mice were 17.0 ± 4.7 and 12.9 ± 2.7, respectively. In distal colon mucosa, the number of PCNA positive cells was almost the same between the each group. Related to cell proliferation, leptin elicits its biological activity through Ob-Rbl, and downstream targets, Akt, Erk and STAT3, may stimulate cell growth signaling with modifying cell cycle-related genes. Thus we examined cell cycle-related genes, p27 and p53. The treatment with 800 ppm pioglitazone up-regulated the mRNA levels of p27 (p<0.05) and p53 (p<0.05), and down-regulated leptin receptor Ob-Rbl (p<0.05) in the colorectal mucosa of KK-*Ay* mice without AOM compared with that of untreated control mucosa (Figure 3).

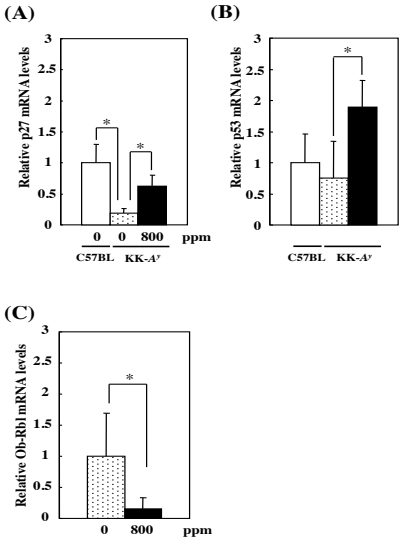

Figure 3. Relative Expression Levels of Cell Cycle-related Genes and Leptin Receptor in Colorectal Mucosa of KK-*Ay* Mice and C57BL/6J Mice. Real-time PCR analysis was performed to obtain p27 (A), p53 (B) and Ob-Rbl (C) mRNA expression levels. GAPDH mRNA was used to normalize the data. White, untreated control C57BL/6J mice. Dotted, untreated KK-*Ay* mice. Black, 800 ppm pioglitazone treated KK-*Ay* mice. Values were set at 1.0 in untreated control. Data are means ± SE (n=4). *, p<0.05.

Discussion

In the present study, pioglitazone treatment decreased the number of AOM-induced ACF in obese KK-*Ay* mice. This suppressive effect of pioglitazone might be explained by involvement of systemic improvement of dysregulated adipocytokine, triglyceride and insulin levels, and increase of mRNA levels of p27 and p53 in the colorectal mucosa of KK-*Ay* mice. This study provided the evidence that pioglitazone could be a useful chemopreventive agent against obesity-associated colorectal cancer.

It has been reported that hypertrophic change of adipocytes evokes dysregulated adipocytokine production (Cowey et al., 2006; van Kruijsdijk et al., 2009). Thus, we examined the size of adipocytes in visceral adipose tissue in KK-*Ay* mice treated with pioglitazone and found the

size to be much smaller than those in untreated control mice. These data are consistent with previous reports that PPARγ, a member of the nuclear receptor superfamily, stimulated preadipocyte differentiation, and reduced the size of adipocytes to the normal size (Schoonjans et al., 1996). Size reduction of adipocyte is suggested to improve insulin resistance along with reduced serum triglyceride levels. In fact, serum levels of triglycerides, insulin and leptin levels were decreased at a dose of 800 ppm pioglitazone, and serum adiponectin was increased. In addition, the expression of LPL was increased in the liver being related to reduction of serum triglyceride levels. It has been reported that the PPAR-responsible elements exist in the promoter region of the LPL gene, and indeed, pioglitazone increased hepatic expression levels of LPL. Moreover, the mRNA expression levels of IL-6, leptin, MCP-1, Pai-1 and TNF-α in visceral adipose were reduced by pioglitazone treatment, and adiponectin tended to be increased in the present study. It has been reported that PPARγ ligand inhibits Ob-Rbl mRNA expression in human hepatic stellate cells (Schoonjans et al., 1996). Several experiments using thiazolidinediones, selective ligands of PPARγ, revealed that PPARγ targets adiponectin, IL-6, MCP-1 and TNF-α and increase adiponectin expression levels but decrease the rest (Iwaki et al., 2003).

The expression of cell cycle-related genes (p27 and p53) in colorectal mucosa of KK-A^y mice was down-regulated compared with those of C57BL/6J mice, which are generally used as non-obese, non-diabetic controls (Figure 3). P27 and p53, which belong to the Cip/Kip family of cyclin-dependent kinase inhibitors, play a key role in cell growth arrest (Polyak et al., 1994). The administration of 800 ppm pioglitazone increased the expression levels of low p27 and p53 mRNA levels observed in the colorectal mucosa of KK-A^y mice. Leptin elicits its biological activity through Ob-Rbl, and downstream targets, Akt, Erk and STAT3, may stimulate cell growth signaling with modifying cell cycle-related genes. It has been reported that STAT3 would play both a positive regulatory role and a negative one for p27 expression (Fukada et al., 1998; Kortylewski et al., 1999). Thus, it is implicated that low p27 and p53 in the obese mice could be due to high serum levels of leptin in part, and that increased expression of p27 and p53 by pioglitazone treatment could due to a decrease of leptin expression. Of note, insulin and insulin-like growth factors are strong growth factors modifying cell cycle-related genes and may affect colorectal ACF development. The serum level of insulin, drastically decreased with pioglitazone, also could explain the effects of pioglitazone on ACF development. The ratio of contribution of the factors, such as adipocytokine, insulin and triglyceride should be revealed in the future.

It is interesting that pioglitazone suppressed AOM-induced ACF development in the upper portion of the colorectum (proximal and middle colon), but not lower portion (distal colon and rectum). To clarify the localized specific effect of pioglitazone, PCNA immunohistochemical staining was conducted in middle and distal colon. As a result, administration of 800 ppm pioglitazone had a tendency to reduce PCNA positive cells in the middle colon in KK-A^y mice. Moreover, there were no significant differences in mRNA levels of p27 and p53 between the middle and distal parts (data not shown). Comparing the numbers of ACF in AOM-treated lean C57BL/6J mice with those of KK-A^y mice, KK-A^y mice increased the number of ACF in the proximal and middle colon (Teraoka et al., 2011). However, the effect of pioglitazone on different portions of the colon in obese mice could not be explained. Further examinations with novel aspects are needed to clarify the different action of pioglitazone on ACF development in the distal and middle colon.

In conclusion, pioglitazone has a potential benefit to suppress AOM-induced ACF development in obese KK-A^y mice in a systematic and direct manner. Pioglitazone also could effectively suppress intestinal polyp development in Min mice (Niho et al., 2003). Thus, pioglitazone might be a good candidate for a chemopreventive agent against obesity-associated colorectal cancer. Meanwhile, a cohort study showed no clear associations between use of pioglitazone and reduced risk of colon cancer incidence in diabetes patients (Ferrara et al., 2011), with the limitation of short periods of follow up, less than 6 years, after the initiation of pioglitazone. Thus, further epidemiological studies with long periods of follow up are desired to evaluate pioglitazone, as a potential chemopreventative agent in humans.

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