研究成果の刊行に関する一覧表

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Suppression of *N*-nitrosobis(2-oxopropyl)amine-induced pancreatic carcinogenesis in hamsters by pioglitazone, a ligand of peroxisome proliferator-activated receptor γ

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Fat intake and obesity are positively correlated with pancreatic cancer in humans. *N*-nitrosobis(2-oxopropyl)amine (BOP) induces pancreatic ductal adenocarcinomas limited to Syrian golden hamsters, other rodents not being susceptible. In the present study, we found markedly high levels of serum triglycerides (TGs) and total cholesterol (TC) in Syrian golden hamsters, but not C57BL/6 mice, ICR mice, F344 rats and Wistar rats. Consistent with this, lipoprotein lipase (LPL) activities in the liver were lower in hamsters compared with mice and rats. To examine effects of pioglitazone, a peroxisome proliferator-activated receptor γ (PPARγ) ligand, on LPL expression, serum lipid levels and pancreatic cancer development, 6-week-old female Syrian golden hamsters were subcutaneously injected with BOP (10 mg/kg body wt) four times in a week and thereafter fed a diet containing 800 p.p.m. pioglitazone for 22 weeks. The treatment elevated LPL mRNA expression in the liver and significantly improved hyperlipidemia with serum levels of TG and TC being decreased to 62 and 71%, respectively, of the control values. Concurrently, the incidence and multiplicity of pancreatic ductal adenocarcinomas were significantly decreased by pioglitazone in comparison with the controls (38 versus 80%, $P < 0.01$ and 0.55 ± 0.15 versus 1.37 ± 0.22, $P < 0.01$, respectively). The suppression rates were grater in invasive adenocarcinomas than non-invasive ones. The incidence of cholangiocellular carcinomas was also reduced. Thus, suppression of pancreatic adenocarcinoma development by pioglitazone is possibly associated with improvement in the serum lipid profile, and hyperlipidemia could be an enhancing factor for development of pancreatic cancer in hamsters.

Introduction

It was estimated that ~220 000 people die of pancreatic cancer worldwide every year (1), this being the fourth leading cause of cancer-related mortality in the USA (2) and the fifth in Japan (3), where pancreatic cancer is steadily increasing in incidence and a 5-year survival rate is extremely low (4). From epidemiological studies, environmental factors like cigarette smoking and dietary habits are risk factors (5), one report implicating a high intake of saturated fat (6). Likewise, cohort studies have indicated that obese individuals are at increased risk of pancreatic cancer (7,8). Generally, progress of pancreatic cancer is very silent, so early detection is extremely difficult. In addition, effective chemotherapeutic and chemopreventive agents against pancreatic cancer have yet to be identified. Therefore, elucidation of causative factors and mechanisms underlying pancreatic carcinogenesis is a high priority.

The Syrian golden hamster is a unique model animal for development of ductal pancreatic cancer with subcutaneous injections of *N*-nitrosobis(2-oxopropyl)amine (BOP) (9), the induced lesions having close similarities to the major form of pancreatic cancer in humans. Point mutations in codon 12 of the *K-ras* gene are frequently observed (10), and expression of the *fragile histidine triad* gene, a tumor suppression gene, is generally abnormal in hamsters (11) as in human cases (12,13). Interestingly, BOP does not induce pancreatic ductal cancers in mice (14) or rats (15) and the reason for the species specificity is not clear. Incidentally, we found the age-dependent hyperlipidemic state in hamsters even when the animals were fed a low-fat standard diet, but not in mice and rats.

Peroxisome proliferator-activated receptor γ (PPARγ), one of the nuclear receptor superfamily of ligand-activated nuclear transcription factors (16,17), is prominently expressed in adipose tissue, and also in other tissues at lower levels (18). One of its biological functions is to regulate adipocyte differentiation (19). Thiazolidinediones (TZDs) are ligands for PPARγ, and one of the TZD derivatives, pioglitazone, has been clinically accepted as an anti-diabetic drug. It is well established that administration of TZDs improves hyperlipidemia and hyperglycemia in animal models (20,21). Furthermore, TZDs have been shown to inhibit the proliferation of various cancers (22–25). We have previously reported that *adenomatous polyposis coli* (*Apc*) gene-deficient mice, *Apc*^{1309} and Min mice, develop hyperlipidemia with down-regulation of lipoprotein lipase (LPL) mRNA and pioglitazone as well as a LPL selective inducer, NO-1886, improves this hyperlipidemia with up-regulation of LPL mRNA and suppresses intestinal polyp formation (26–28).

In the present study, we examined serum lipid levels and LPL activity in the livers of hamsters, mice and rats on a 5% fat standard diet, and showed the hyperlipidemic state and lowered hepatic LPL activity in the hamsters. Moreover, pancreatic cancer development was significantly suppressed with improvement of hyperlipidemia by pioglitazone. Based on these data, the role of hyperlipidemia in pancreatic carcinogenesis is discussed.

Abbreviations: Apc, adenomatous polyposis coli; BOP, *N*-nitrosobis(2-oxopropyl)amine; IL, interleukin; LPL, lipoprotein lipase; MMP, matrix metalloproteinase; PCR, polymerase chain reaction; PPARγ, peroxisome proliferator-activated receptor γ; RT, reverse transcription; TC, total cholesterol; TG, triglyceride; TZD, thiazolidinedione.

Materials and methods

Animals and chemicals

Female Syrian golden hamsters were obtained from Japan SLC (Shizuoka, Japan) when 5 weeks old and weighing ~80 g and acclimated to laboratory conditions for a week. They were housed two or three per plastic cage, with sterilized softwood chips as bedding, in an air-conditioned animal room, on a 12-h light–dark cycle. Powdered CE-2 (CLEA Japan, Shizuoka, Japan) was employed as a standard basal diet, in which fat is contained at ~5%. Body weights were measured weekly and food consumption twice a week. Food and water were available *ad libitum*. Female C57BL/6 mice, female F344 rats, female ICR mice and female Wistar rats were obtained from Japan SLC, and their care and food administration conformed to the hamster case. The experimental protocols were approved by the Institutional Ethics Review Committee for Animal Experimentation. BOP was obtained from Nacalai Tesque (Kyoto, Japan) and pioglitazone, (±)-5-[4-[2-(5-ethyl-2-pyridyl)ethoxy]benzyl]thiazolidine-2,4-dione monohydrochloride, was kindly provided by Takeda Pharmaceutical Co., Ltd (Osaka, Japan), and well mixed with powdered CE-2 at a concentration of 800 p.p.m.

Measurement of serum lipid levels in animals

Subgroups of 5–13 hamsters were killed at 6 and 30 weeks of age, and blood samples were collected from the heart under diethyl ether anesthesia after overnight fasting. C57BL/6 mice and F344 rats (five to eight animals per group) were also killed at 6 and 30 weeks of age and blood samples similarly obtained. Blood samples from ICR mice and Wistar rats at 6 and 25 weeks of age were also used. The levels of triglyceride (TG) and total cholesterol (TC) in the serum were measured as reported previously (29).

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Measurement of LPL activity in the liver

Liver samples from 6-week-old Syrian golden hamsters, C57BL/6 and ICR mice and F344 and Wistar rats were used. LPL activity was determined using a triolein-tri[1-^{14}C]oleoyl glycerol emulsion substrate as previously reported (30).

Analysis of LPL expression in the livers of hamsters by reverse transcription–polymerase chain reaction

Primers for LPL in hamsters were designed from cDNA sequences that matched perfectly between the mouse (accession number, NM_008509) and rat (NM_012598), and the cDNA sequence of hamster LPL in the open reading frame was analyzed by reverse transcription (RT)–polymerase chain reaction (PCR) and direct sequencing using an ABI310 PRIZM DNA Sequencer (Applied Biosystems, Foster City, CA). A high degree of sequence similarity was observed with mouse and rat, homologies being 91% and >95% for the nucleotide and amino acid sequences, respectively. The analyzed cDNA sequence was registered at the DNA Data Bank of Japan; accession number, AB194713. Liver samples were taken from 6-week-old Syrian golden hamsters and stored at −80°C. Total RNA was extracted from the liver samples using Trizol reagent (Invitrogen, Co., Carlsbad, CA). After RNA purification and subsequent DNase I treatment, aliquots of total RNA (2.5 μg) were subjected to the RT reaction with nonamer random primers in a final volume of 50 μl using an Omniscript RT kit (Qiagen, Hilden, Germany). PCR amplification was performed in a final volume of 50 μl with aliquots of cDNA (150 ng) and iTaq DNA polymerase (Bio-Rad Laboratories, Hercules, CA) using a PTC-200 Peltier thermal cycler (MJ Research, Waltham, MA). The primers used were selected from the common sequences among hamster, mouse, rat and human cDNA of LPL and β-actin—5′-primer: ATTTGCCCTAAGGACCCCTG and 3′-primer: GCACCCAACTCTCATACATTCC (product size, 157 bp) for LPL and 5′-primer: ACGAGGCCCAGAGCAAGAGA and 3′-primer: TGGCTGGGGTGTTGAAGGTC (product size, 228 bp) for β-actin. The cycling conditions were as follows: 95°C for 3 min, 33 cycles (for LPL) and 28 cycles (for β-actin) of 94°C for 5 s, 60°C for 20 s and 72°C for 30 s and a 10-min cycle at 72°C. The products were analyzed by 3% agarose gel electrophoresis with ethidium bromide staining. Quantitative real-time RT–PCR was performed using a PTC-200 DNA engine cycler equipped with a CFD-3220 Opticon 2 detector (MJ Research) for fluorescence detection, and SYBR Green I (Bio-Whittaker Molecular Applications, Rockland, ME) was used as a fluorescence dye. The primers employed were as described above, with cycling conditions as follows: 95°C for 3 min, 45 cycles of 94°C for 5 s, 60°C for 20 s, 72°C for 30 s and 79°C for 2 s. The fluorescence intensity of SYBR Green I was measured at 79°C with every cycle. Each PCR product was subcloned into the TA cloning plasmid vector using the pTARGET mammalian expression system (Promega Co., Madison, WI) and a JM109 Transformation kit (Nippon Gene, Co., Ltd, Toyama, Japan). To determine the copy numbers of cDNA for LPL and β-actin, their plasmids were cumulatively added to PCR samples as templates in the range of 10^2–10^8 copies. Finally, the PCR products were analyzed by 3% agarose gel electrophoresis with ethidium bromide staining to confirm the correct sizes.

Carcinogenicity study and histopathological examination

Sixty hamsters at 6 weeks old were injected subcutaneously with BOP four times (on days 1, 3, 5 and 7) at a dose of 10 mg/kg body wt, a further 20 hamsters receiving saline as vehicle controls. From 1 week after the last BOP treatment, one half of each group was given basal diet and the other diet containing 800 p.p.m. of pioglitazone for 22 weeks. The dose was chosen from our previous study in mice (27) and preliminary study in hamsters (data not shown). At the killing time point at 30 weeks of age, all surviving animals were anesthetized with diethyl ether and blood samples were collected from the heart. At autopsy, the pancreas, heart, lungs, kidneys, liver and bile duct were carefully examined macroscopically. The heart, lungs, kidneys, liver and bile duct were fixed in 10% phosphate-buffered formalin (pH 7.4). Each pancreas was carefully dissected from surrounding tissue and fixed after spreading on filter paper. All paraffinized organs were sectioned and stained with hematoxylin and eosin for assessment of histopathological features, as described previously (31,32).

Statistical analysis

The significance of differences in the incidences of tumors was analyzed by the χ^2 test. Variation in other data was evaluated by the Student's *t*-test. A *P* value of <0.05 was regarded as significant.

Results

Hyperlipidemic state in hamsters

Serum lipid levels of hamsters were compared with those of mice and rats. As shown in Table I, levels of TG in the serum of Syrian golden hamsters fed a standard diet were markedly high, being 322 ± 41 mg/dl at 6 weeks old, which were 7-fold of those in C57BL mice and 2.8-fold of those in F344 rats. Serum TC levels were also high in Syrian golden hamsters, being 179 ± 6 mg/dl at 6 weeks old, which were 2.1-fold of those in C57BL mice and 1.6-fold of those in F344 rats. The serum TG and TC in 30-week-old hamsters were also in the levels of hyperlipidemia being similar or higher than those in 6-week-old hamsters. On the other hand, serum TG and TC in C57BL mice and F344 rats were not increased at 30 weeks of age. Similarly to C57BL mice and F344 rats, low levels of TG and TC were observed in other strains of rats and mice, such as ICR mice and Wistar rats.

In addition to the hyperlipidemic state in blood, histopathological evaluation revealed that all hamsters at 30 weeks of age suffered from steatosis in the livers (Figure 1).

Low activity of LPL in hamsters

LPL is a key enzyme to decompose TGs. A low activity of LPL could be one of the causes of hyperlipidemia. LPL activity in the liver of hamsters was then compared with those of mice and rats (Table II). LPL activities in Syrian golden hamsters at 6 weeks of age were 119 ± 36 nmole/min/wet g tissue, whereas those in C57BL mice and F344 rats were significantly higher being 5.1-fold and 3.1-fold, respectively, of the value in hamsters. LPL activities in ICR mice and Wistar rats at 6 weeks of age were also significantly higher than those in hamsters.

Effects of pioglitazone on the hyperlipidemic state and cancer development in BOP-treated hamsters

To examine the effect of improvement of hyperlipidemia on pancreatic carcinogenesis in hamsters, hamsters were treated with a

Table I. Serum lipid levels of hamsters, mice and rats

Species	Strain	Age (weeks)	No. of animals	TG (mg/dl)	TC (mg/dl)
Hamster	Syrian golden	6	13	322 ± 41[a]	179 ± 6
		30	14	363 ± 26	248 ± 13[b]
Mouse	C57BL	6	8	46 ± 5[c]	86 ± 6[c]
		30	5	43 ± 6[c]	102 ± 12[c]
	ICR	6	5	63 ± 11[c]	113 ± 7[c]
		25	5	64 ± 7[c]	104 ± 8[c]
Rat	F344	6	8	115 ± 11[c]	109 ± 7[c]
		30	5	39 ± 4[bc]	92 ± 2[c]
	Wistar	6	5	102 ± 16[c]	92 ± 2[c]
		25	5	61 ± 9[c]	93 ± 5[c]

[a]Data are mean ± SE values.
[b]Significantly different from the value at 6 weeks of age, at $P < 0.0005$.
[c]Significantly different from the corresponding value in hamsters, at $P < 0.0005$.

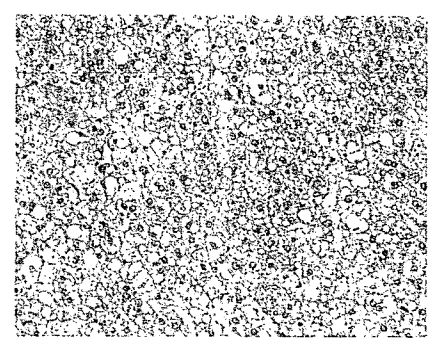

Fig. 1. Liver steatosis in hamster. Histopathological appearanceof tissue sections stained with hematoxylin and eosin—note liver steatosis at 30 weeks of age.

pancreatic carcinogen, BOP, and then fed a diet containing 800 p.p.m. pioglitazone. Administration of pioglitazone for 22 weeks after treatment with BOP did not affect the behavior of hamsters, but pioglitazone brought about a slight increase of food intake: the average food intake (g/day/animal) was 10.4 ± 1.2 in the BOP + 800 p.p.m. pioglitazone group and 9.6 ± 0.7 in the BOP + basal diet group ($P < 0.005$). The final body weight was statistically higher in the BOP + 800 p.p.m. pioglitazone group compared with in the BOP + basal diet group (219.5 ± 20.9 versus 202.3 ± 19.9, $n = 29$ each, $P < 0.005$). As shown in Figure 2A and B, serum levels of TG and TC in 30-week-old hamsters were significantly decreased by pioglitazone to 62% ($P < 0.001$) and 71% ($P < 0.001$), respectively, of the values of the basal diet group. Severe hepatic steatosis observed in the entire liver in the basal diet group was also ameliorated by the pioglitazone treatment to mild (data not shown). Furthermore, as shown in Figure 3A, expression levels of LPL mRNA in the liver were elevated by treatment with 800 p.p.m. of pioglitazone, quantitative real-time RT–PCR assays demonstrating ~1.7-fold higher values than for the group without pioglitazone (Figure 3B).

Pancreatic lesions were histopathologically diagnosed as atypical hyperplasias, non-invasive adenocarcinomas and invasive adenocarcinomas, and the incidence and multiplicity data are summarized in Tables III and IV, respectively. The effective number was defined as the number of animals that survived until 30 weeks of age. As shown in Table III, the incidence of the adenocarcinomas induced by BOP was lower in the group treated with 800 p.p.m. pioglitazone than that in the control group: 38 versus 80% ($P < 0.01$). Multiplicity of the adenocarcinomas was also decreased by pioglitazone: 0.55 ± 0.15 versus 1.37 ± 0.22 ($P < 0.01$) (Table IV). Especially, the incidence and multiplicity of invasive adenocarcinomas were significantly lower in the pioglitazone-treated group than the control group being 31 versus 73% ($P < 0.01$) and 0.38 ± 0.12 versus 1.03 ± 0.16 ($P < 0.01$), respectively, whereas those of non-invasive adenocarcinomas did not significantly differ between the two groups.

In addition to pancreatic ductal tumors, tumors in the bile duct, liver, lungs and kidneys have been reported to be induced by BOP in hamsters (32). In the present study, cholangiocellular adenomas and carcinomas were observed in the BOP-treated group at incidences of 3 and 47%, respectively (Table V). These cholangiocellular tumors were developed both in intra- and extrahepatic bile ducts. With administration of pioglitazone, their development was also markedly suppressed (Table V). In contrast, lung adenomas were not affected, the incidences being 59% (17/29) and 53% (16/30) with and without pioglitazone, respectively. Renal mesenchymal tumors and hepatocellular carcinomas were observed only in the BOP-alone group, at incidences of 10% (3/30) and 3% (1/30), respectively, but their incidences were not significantly different from those in the BOP + pioglitazone group (0/29 and 0/29, respectively). Tumors in the pancreatic duct, bile duct, liver, lungs and kidneys were not observed in the saline vehicle ($n = 10$) or pioglitazone group hamsters ($n = 10$) without the BOP treatment. BOP treatment induced lipomatosis in the pancreas with its toxic effects. In addition, lipomatosis in the pancreas was observed in the pioglitazone-treated groups with and without BOP, however, no toxic signs were seen in the pioglitazone-alone group.

Discussion

Our present investigation unequivocally demonstrated a hyperlipidemic state in hamsters on a 5% fat standard diet, but not in mice and rats.

Table II. LPL activities in the liver of 6-week-old hamsters, mice and rats

Species	Strain	No. of animals	LPL activity (nmole/min/wet g tissue)
Hamster	Syrian golden	5	119 ± 36[a]
Mouse	C57BL	5	605 ± 72[b]
	ICR	5	375 ± 14[b]
Rat	F344	5	370 ± 56[c]
	Wistar	5	305 ± 21[c]

[a]Data are mean ± SE values.
[b]Significantly different from the corresponding value in hamsters, at $P < 0.005$.
[c]Significantly different from the corresponding value in hamsters, at $P < 0.05$.

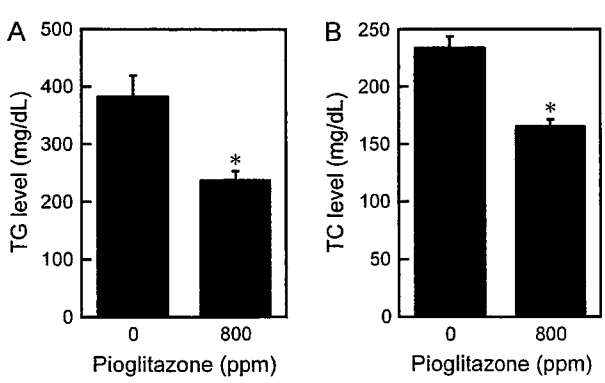

Fig. 2. Decrease in serum lipid concentrations by pioglitazone in hamsters. Levels of serum TG and TC in BOP-treated hamsters at 30 weeks of age given diet with or without 800 p.p.m. of pioglitazone for 22 weeks are shown in (**A** and **B**), respectively ($n = 30$). Data are mean ± SE values. Asterisks indicate a significant difference from the controls at $P < 0.001$.

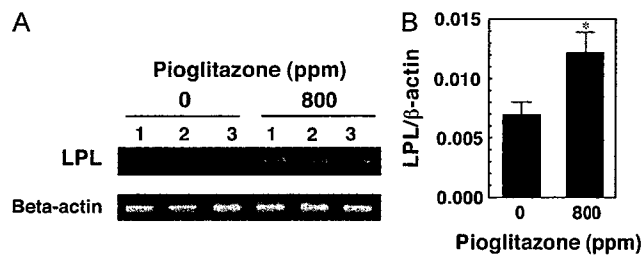

Fig. 3. Elevated expression of LPL mRNA by pioglitazone in hamsters. (**A**) RT–PCR analysis of LPL mRNA in the livers of hamsters at 30 weeks of age given diet with or without 800 p.p.m. of pioglitazone for 22 weeks. Three hamsters each were used for the RT–PCR analysis with and without pioglitazone treatment, respectively. (**B**) Quantitative results of the LPL mRNA expression levels by real-time RT–PCR. Data are mean ± SE values ($n = 15$). Asterisks indicate a significant difference from the control at $P < 0.05$. β-Actin was used as an internal control. Copy numbers of LPL and β-actin were determined using plasmids in which the PCR products were inserted as templates.

Table III. Effects of pioglitazone treatment on the incidences of BOP-induced pancreatic lesions in hamsters[a]

Group	Effective number of animals	Number of animals with lesions			
		AH	NIAC	IAC	NIAC + IAC
BOP	30	2 (7)[b]	8 (27)	22 (73)	24 (80)
BOP/800 p.p.m. pioglitazone	29	2 (7)	6 (21)	9 (31)[c]	11 (38)[c]

AH, atypical hyperplasia; NIAC, non-invasive adenocarcinoma and IAC, invasive adenocarcinoma.
[a]Hamsters were fed a basal diet or a diet containing a PPARγ agonist for 22 weeks.
[b]Percentage in parentheses.
[c]$P < 0.01$ compared with the basal diet group.

Table IV. Effects of pioglitazone treatment on the multiplicity of BOP-induced pancreatic lesions in hamsters[a]

Group	Effective number of animals	Number of lesions per animal			
		AH	NIAC	IAC	NIAC + IAC
BOP	30	0.07 ± 0.05[b]	0.30 ± 0.10	1.03 ± 0.16	1.37 ± 0.22
BOP/800 p.p.m. pioglitazone	29	0.10 ± 0.08	0.17 ± 0.07	0.38 ± 0.12[c]	0.55 ± 0.15[c]

AH, atypical hyperplasia; NIAC, non-invasive adenocarcinoma and IAC, invasive adenocarcinoma.
[a]Hamsters were fed a basal diet or a diet containing a PPARγ agonist for 22 weeks.
[b]Data are mean ± SE values.
[c]$P < 0.01$ compared with the basal diet group.

Table V. Effects of pioglitazone treatment on the incidence of BOP-induced bile duct tumors in hamsters[a]

Group	Effective number of animals	Number of animals with lesions	
		Cholangiocellular adenoma	Cholangiocellular carcinoma
BOP	30	1 (3)[b]	14 (47)
BOP + 800 p.p.m. pioglitazone	29	0 (0)	0 (0)[c]

[a]Hamsters were fed a basal diet or a diet containing a PPARγ agonist for 22 weeks.
[b]Percentage in parentheses.
[c]$P < 0.0001$ compared with the basal diet group.

It was also observed that 30-week-old hamsters suffered from steatosis in the liver. Furthermore, the LPL activity in the liver of hamsters was significantly lower than those of the other rodents. Dietary administration of pioglitazone, a PPARγ agonist, decreased serum lipid levels of TG and TC, elevated LPL mRNA expression in the liver and simultaneously suppressed pancreatic ductal adenocarcinoma development, especially the incidence and multiplicity of invasive adenocarcinomas, in BOP-treated hamsters. These results suggest that hyperlipidemia in hamsters may be an enhancing factor for pancreatic ductal cancer development.

This study provides an important insight into the etiology of pancreatic cancer, supporting the epidemiological finding that fat intake and obesity might increase pancreatic cancer risk (6–8). Malfunction of LPL is one reason for hyperlipidemia (33) and several polymorphisms resulting in lowered activity have been reported to cause primary hypertriglyceridemia in humans (34). From the results of cloning hamster LPL, hamster-specific amino acid changes were found for 4 of 474 amino acids, corresponding to the open reading frame of LPL, but not at reported polymorphism positions, and the amino acid sequence at the active site appears fully conserved in hamsters. It has been reported that transcriptional induction of LPL is mediated via binding of a heterodimeric complex, consisting of PPAR and the retinoid X receptor, to the functional peroxisome proliferator response element sequence in the promoter of the LPL gene (33). Our previous investigations demonstrated that administration of pioglitazone can up-regulate expression of LPL mRNA and suppress dose dependently both hyperlipidemia and intestinal polyp formation in *Apc*-deficient mice (26,27). An increase in expression of LPL mRNA was similarly shown in hamsters after dietary administration of pioglitazone in the present study, with a decrease of serum lipid concentrations and significant suppression of pancreatic cancers in BOP-treated hamsters. Thus, it is probable that these are causally linked. However, it has been reported that pioglitazone inhibits pancreatic cancer cell growth and invasiveness *in vitro* (35), so that the direct suppressive effects on cancer cell itself might also be involved. There is a LPL selective inducer, NO-1886, that up-regulates LPL mRNA expression without PPARγ activation, and we have demonstrated the suppressive effect of NO-1886 on intestinal polyp formation in *Apc*-deficient mice (28). To exclude the effect of PPARγ activation and clarify the involvement of LPL in suppression of pancreatic cancer development, further investigation using NO-1886 is warranted.

Hibernating animals such as hamsters exhibit an obese state by deposition of adipose tissues (36) and cytokines secreted from adipose tissues could be involved in down-regulation of LPL. Skeletal muscle LPL protein levels are reduced by adipocyte tissue hypertrophy (37) and it is known that interleukin (IL)-6 and leptin are released from adipocytes, levels being elevated in obese subjects (38). The molecular function of IL-1β is positively correlated with expression of leptin (39) and there have been reports that IL-6 and IL-1β can reduce LPL activity (40,41). These adipocytokines enhance the invasiveness of pancreatic cancer, and leptin strongly stimulates the secretion of matrix metalloproteinase-2 (42), which has a primary role in pancreatic cancer cell invasion (43) and is significantly activated in metastatic lesions (44). In the present study, the mass of adipose tissues in hamsters was decreased 38% by pioglitazone treatment in comparison with the controls (data not shown). It is thus suggested that the strong suppression of invasive adenocarcinoma might have been caused by reduced secretion of adipocytokines, due to this decrease of adipose tissue.

In addition, our data indicate that pioglitazone also significantly suppresses the development of cholangiocellular carcinomas, but not lung adenomas. Pancreatic ductal adenocarcinomas and cholangiocellular carcinomas in BOP-treated hamsters have certain genetic characteristics in common; for example, aberrant transcription of the *fragile histidine triad* gene is observed in both (11,45). Thus, the influence of pioglitazone might be similar for the two types of carcinoma, one arising from ducts in the pancreas and the other from bile ducts. Interestingly, it has been reported that 4-phenylbutyl isothiocyanate suppresses pancreatic cancers and lung tumors, but enhances liver tumorigenesis in BOP-treated hamsters (46). Phenethyl isothiocyanate and a cyclooxygenase inhibitor, nimesulide, also suppress BOP-induced hamster pancreatic cancers and lung tumors, but do not affect liver tumors (32). Thus, the inhibitory mechanisms of pioglitazone on pancreatic cancer may be different from those of these agents.

To conclude, the present study demonstrated a hyperlipidemic state in hamsters, and its improvement with pioglitazone accompanied by suppression of the development of invasive pancreatic adenocarcinomas. The inhibitory effects appeared more remarkable than with a cyclooxygenase-2 inhibitor, nimesulide (32), soybean trypsin inhibitor (47) and phenethyl isothiocyanate (48), so that anti-hyperlipidemic drugs may deserve more consideration as candidate chemopreventive agents active against pancreatic cancer.

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Improvement of hyperlipidemia by indomethacin in Min mice

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***Apc* gene-deficient Min and *Apc*^{1309} mice feature a hyperlipidemic state with a markedly low expression level of lipoprotein lipase (LPL) compared to their wild-type counterparts. We previously showed that induction of LPL mRNA by peroxisome proliferator-activated receptor (PPAR) α and γ agonists or an LPL selective inducer suppresses both high serum lipid levels and intestinal polyp formation in these model animals. Since the general cyclooxygenase inhibitor, indomethacin, is known to suppress intestinal tumor development, but not to affect serum lipids, its influence in Min mice was here investigated. Treatment with 2.5, 5 and 10 ppm indomethacin in the diet for 14 weeks from 6 weeks of age caused significant dose-dependent reduction in serum triglycerides, along with a reduction in the numbers of intestinal polyps to 25% of the untreated control value. LPL mRNA levels in the liver were slightly increased by indomethacin treatment. We further performed oligonucleotide microarray analysis and quantitative PCR analysis and found 8 lipid metabolism-related genes, regulated by sterol regulatory element binding protein-1c, to be modulated by indomethacin-treatment in the Min mouse liver. Furthermore, TNFα was downregulated. These results indicate that indomethacin might suppress intestinal tumor formation together with a hyperlipidemic state by regulating LPL and other lipid metabolic factors.**

Key words: *Apc*; Min; hyperlipidemia; colon cancer; NSAIDs

Epidemiological studies have suggested that mortality and morbidity rates of colon cancer are increasing in developed countries.[1,2] Consumption of a high fat diet is a considerable risk factor and serum lipid levels have been indicated to be positively associated with colon carcinogenesis.[3–5]

Enormous efforts have been made to develop colorectal cancer chemopreventive agents, and nonsteroidal anti-inflammatory drugs (NSAIDs), such as indomethacin and aspirin, are useful candidates from experimental, epidemiological and clinical findings.[6–10] Indomethacin is a conventional NSAID which has long been clinically employed to target inflammation. It also possesses potent chemopreventive activity against intestinal tumor development in animal models, and has been tested by clinical trial in familial adenomatous polyposis (FAP) patients to reduce the intestinal polyps from which colorectal cancers develop.[7,11] The molecular mechanisms underlying its protective effects are considered mainly due to inhibition of cyclooxygenase-1 (COX-1) and COX-2 activity, involved in prostanoid synthesis.[12]

Recently, we clearly demonstrated a hyperlipidemic state in the *Apc*^{1309} and Min strains of *Apc*-deficient mice, animal models of human FAP, because of depression of lipoprotein lipase (LPL) mRNA in the livers and small intestines.[13–15] LPL, known to be transcriptionally regulated by peroxisome proliferator-activated receptor (PPAR), catalyzes the hydrolysis of triglycerides into fatty acids and monoacylglycerol.[16] Experimental induction of LPL mRNA by a PPARα agonist, bezafibrate, and a PPARγ agonist, pioglitazone, was found to suppress both the hyperlipidemic state and intestinal polyp formation in these mice.[13,14] Furthermore, an LPL selective inducer without PPAR agonistic activity, NO-1886, also suppressed hyperlipidemia and intestinal polyp formation in Min mice.[15] These results thus suggested that suppression of serum lipid levels might contribute to the reduction of intestinal polyp formation in Min mice.

Understanding of NSAID's chemopreventive activity is based on extensive studies. However, effects of NSAIDs on LPL expression and serum lipid levels which may contribute to intestinal carcinogenesis have hitherto not been reported. Thus, in the present study, we aimed to clarify whether indomethacin might influence the hyperlipidemic state in Min mice, and indeed found strong serum lipid-lowering effects along with moderate LPL induction. We also performed DNA microarray analysis and identified several expression changes in genes regulating serum lipid levels. On the basis of these data, a novel mechanism of chemopreventive activity by indomethacin is proposed.

Material and methods

Animals and chemicals

Female C57BL/6-*Apc*$^{Min/+}$ mice (Min mice), 5 weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, ME) and genotyped by the method reported previously.[13] Heterozygotes of the Min strain and wild-type mice were acclimated to laboratory conditions for 1 week. Three to five animals were housed per plastic cage, with sterilized softwood chips as bedding, in a barrier-sustained animal room, air-conditioned at 24°C ± 2°C and 55% humidity, on a 12 hr light/dark cycle. Indomethacin was purchased from Sigma Chemical (St. Louis, MO) and well mixed at concentrations of 2.5, 5 and 10 ppm with AIN-76A powdered basal diet (CLEA Japan, Tokyo, Japan).

Experimental protocol for Min mice treated with indomethacin

Ten to 13 female Min mice in each group were given 2.5, 5 or 10 ppm indomethacin in the diet for 14 weeks from 6 to 20 weeks of age, while the control group received basal diet. Food and water were available *ad libitum*. The animals were observed daily for clinical signs and mortality, and body weights and food consumption were measured weekly. At the sacrifice time points, mice were anesthetized with ether, and blood samples were collected from the abdominal vein. The levels of serum triglycerides and total cholesterol were measured as previously reported.[13] In addition, very low-density lipoprotein cholesterol (VLDL-C), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were assessed by HPLC.[17] The liver, kidneys, heart and spleen were weighed and tissue samples from the liver of mice were rapidly deep-frozen in liquid nitrogen and stored at −80°C.

The stomach and intestinal tract were removed, filled with 10% buffered formalin and separated into the stomach, small intestine,

Abbreviations: COX, cyclooxygenase; FAP, familial adenomatous polyposis; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LPL, lipoprotein lipase; NSAID, non-steroidal anti-inflammatory drug; PPAR, peroxisome proliferator-activated receptor; SREBP, sterol regulatory element binding protein; VLDL-C, very low-density lipoprotein cholesterol.

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TABLE I – SUPPRESSION OF INTESTINAL POLYP DEVELOPMENT IN MIN MICE BY INDOMETHACIN

Dose (ppm)	No of mice	No. of polyps/mouse				
		Small intestine				
		Proximal	Middle	Distal	Colon	Total
0	8	14.0 ± 3.0[1]	25.8 ± 5.5	34.5 ± 7.3	0.9 ± 0.4	75.1 ± 15.6
2.5	9	11.6 ± 1.4 (83)[2]	13.4 ± 4.3 (53)	20.7 ± 4.4 (60)	0.7 ± 0.3 (76)	46.3 ± 8.3 (62)
5	8	11.3 ± 2.8 (80)	6.9 ± 1.8 (27)[4]	8.9 ± 3.4 (26)[4]	0.5 ± 0.2 (57)	27.5 ± 4.1 (37)[3]
10	8	11.0 ± 2.0 (79)	4.4 ± 1.1 (17)[4]	2.6 ± 0.7 (8)[4]	0.6 ± 0.3 (71)	18.6 ± 3.5 (25)[4]

[1]Data are means ± SE.–[2]Numbers in parentheses are percentages of the control basal diet values.–[3]Significantly different from the basal diet group at $p < 0.05$.–[4]Significantly different from the basal diet group at $p < 0.01$.

cecum and colon. The small intestine was divided into the proximal segment (4 cm in length), and proximal (middle) and distal halves of the remainder. All segments were opened longitudinally and fixed flat between filter paper in 10% buffered formalin. The numbers and sizes of polyps, and their distributions in the intestine were assessed with a stereoscopic microscope. The stomach was embedded in paraffin, sectioned and stained with hematoxylin and eosin.

The experiments were conducted according to the "Guidelines for Animal Experiments in the National Cancer Center" of the Committee for Ethics of Animal Experimentation of the National Cancer Center.

cDNA microarray analysis

Total RNA was isolated from livers of Min and the wild-type mice treated with or without 10 ppm indomethacin in the diet for 14 weeks ($n = 3$ each) and cDNA microarray analysis was performed using GeneChip Murine Genome U74A V.2 Arrays® (Affimetrix, Santa Clara, CA), as reported previously.[18] The microarray chip covers 12,488 genes. Altered expression levels were concluded to be significant when more than 2-fold increase or decrease to less than 1/2 of their expression level were noted between indomethacin-treated and -untreated groups.

Quantitative real-time PCR analysis

Total RNA from liver samples of mice was isolated from tissues using Isogen (Nippon Gene, Tokyo, Japan), treated with DNase I (Invitrogen, Carlsbad, CA) and applied at 3 μg aliquots in a final volume of 20 μl for synthesis of cDNAs using an Omniscript RT Kit (Qiagen, Hilden, Germany) and an oligo(dT) primer. Quantitative real-time PCR was carried out using a DNA Engine Opticon™2 (MJ Japan, Tokyo, Japan) with SYBR Green Realtime PCR Master Mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Primers for LPL,[19] thyroid hormone responsive SPOT14 homolog (SPOT14; Ref. 20), ATP citrate lyase (forward: 5′-GGGAGAAGTTGGGAAGACCA-3′; reverse: 5′-AGGAGGAAGTTGGCAGTGTG-3′), lanosterol synthase (forward: 5′-GCTGGCTTCTTCACTGCTTC-3′; reverse: 5′-TGGCTGCTCTAACTCCCTCA-3′), retinal dehydrogenase 11 (forward: 5′-CAGCCTCATCTACCTCCACA-3′; reverse: 5′-GAGAGCATACCCCCAAAGTC-3′), glycerol kinase (forward: 5′-GGAGACCAGCCGTGTTAAGC-3′; reverse: 5′-GTCCACTG CTCCCACCAATG-3′), 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) synthase 2 (forward: 5′-GCCCAGCAGAGGTTT TCTAC-3′; reverse: 5′-AGGCACAGGGAGTTGATGTC-3′), monoglyceride lipase (forward: 5′-AACCACTAAGCCCCAGTTCC-3′; reverse: 5′-GCAGGATGTGAGCAGAGCAC-3′), acyl-CoA thioesterase 1 (forward: 5′-CCCCTGTGACTATCCTGAGA-3′; reverse: 5′-CTCTTCCAGTTGTGGTCGTC-3′), TNFα,[21] and GAPDH (forward: 5′-TTGTCTCCTGCGACTTCA-3′; reverse: 5′-CACCAC-CCTGTTGCTGTA-3′) were employed. To assess the specificity of each primer set, amplicons generated from the PCR reaction were analyzed for melting curves and also by electrophoresis on 2% agarose gels.

Statistical analysis

The results were expressed as mean ± SE values, and statistical analysis was performed with Dunnett's multiple comparison test and Student's *t* test. Differences were considered to be statistically significant with *p* values less than 0.05.

Results

Suppression of intestinal polyp development in Min mice by indomethacin

Treatment with 2.5–10 ppm indomethacin in the diet for 14 weeks did not affect food intake or clinical signs of Min and the wild-type mice. Final body weights in the groups treated with 0, 2.5, 5 and 10 ppm were 23.4 ± 1.3, 25.3 ± 1.4, 25.4 ± 0.8 and 26.8 ± 1.6 g, respectively. There were no observable adverse effects of indomethacin treatment on any organ weights.

Table I shows data for number and distribution of intestinal polyps in the basal diet and indomethacin-treated groups of Min mice. Almost all polyps were located in the small intestine, with only a few in the colons. Treatment with 2.5, 5 and 10 ppm indomethacin dose-dependently reduced total numbers of polyps to 62%, 37% ($p < 0.05$) and 25% ($p < 0.01$) of the untreated control value, respectively. With 10 ppm indomethacin, the numbers of polyps developing in the proximal, middle and distal parts of the small intestine were reduced to 79%, 17% ($p < 0.01$) and 8% ($p < 0.01$) of the untreated control values, respectively. There was a tendency for reduced colon polyp formation in the indomethacin-treated Min mice (Table I). Administration of 5 and 10 ppm indomethacin significantly reduced the numbers of polyps more than 1.5 and 0.5 mm in diameter, respectively (data not shown). Since gastric damage has been observed in humans with indomethacin treatment,[7] the stomach was histopathologically examined, but no adverse effects of indomethacin were apparent.

Improvement of hyperlipidemia in Min mice by indomethacin

As reported previously,[13–15] a hyperlipidemic state was observed in Min mice fed the basal diet at 20 weeks of age (Fig. 1). Serum levels of triglycerides were 487 ± 66 mg/dl and those of total cholesterol were 119 ± 12 mg/dl. VLDL-C and LDL-C levels of Min mice were higher than those of wild-type mice, and the HDL-C level of Min mice was lower. Administration of indomethacin at doses of 2.5, 5 and 10 ppm dramatically decreased serum levels of triglycerides to 28%, 15% and 10% of the untreated control Min mice value, respectively ($p < 0.01$) (Fig. 1*a*). Furthermore, treatment with 2.5, 5 and 10 ppm indomethacin also decreased total cholesterol levels to 76% ($p < 0.05$), 75% ($p < 0.01$) and 75% of the control value (Fig. 1*b*), while VLDL-C and LDL-C levels were reduced to 7–12% and 24–36% of the untreated control values with 2.5–10 ppm indomethacin, respectively (Figs. 1*c* and 1*d*). In contrast, HDL-C levels were increased almost to the wild-type value at the doses of 2.5–10 ppm (Fig. 1*e*). In the wild-type mice, administration of 2.5–10 ppm indomethacin did not affect the levels of serum triglycerides and cholesterol.

Change of hepatic gene expression caused by indomethacin-treatment

To clarify the mechanisms that suppress hyperlipidemic state by indomethacin, we analyzed mRNA levels for LPL by using quantitative real-time PCR. Consistent with our previous reports,[13–15] LPL mRNA levels in the livers of Min mice were

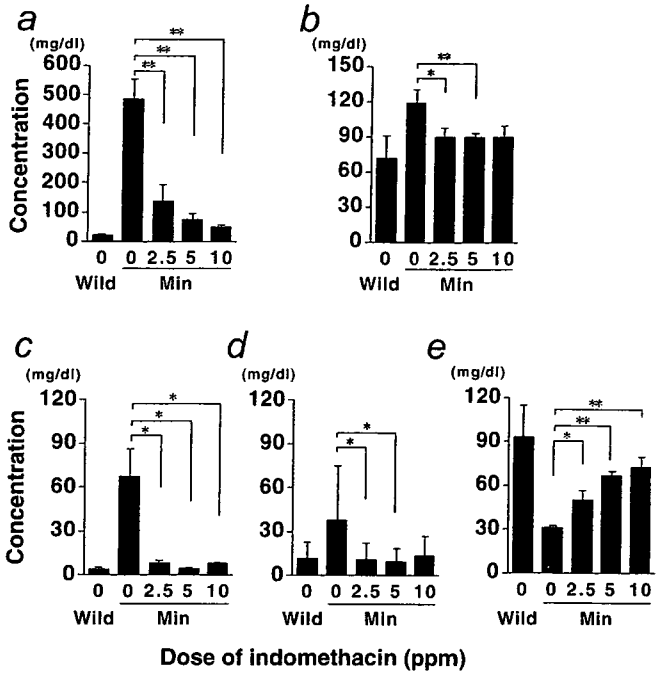

FIGURE 1 – Suppression of serum lipid levels in Min mice by indomethacin. Values for serum levels of triglyceride (*a*), total cholesterol (*b*), VLDL-C (*c*), LDL-C (*d*), and HDL-C (*e*) in female Min mice given basal diet or diet containing indomethacin at doses of 2.5, 5 and 10 ppm for 14 weeks and female wild-type mice are shown. Data are means; *bars*, SE. $*p < 0.05$, $**p < 0.01$.

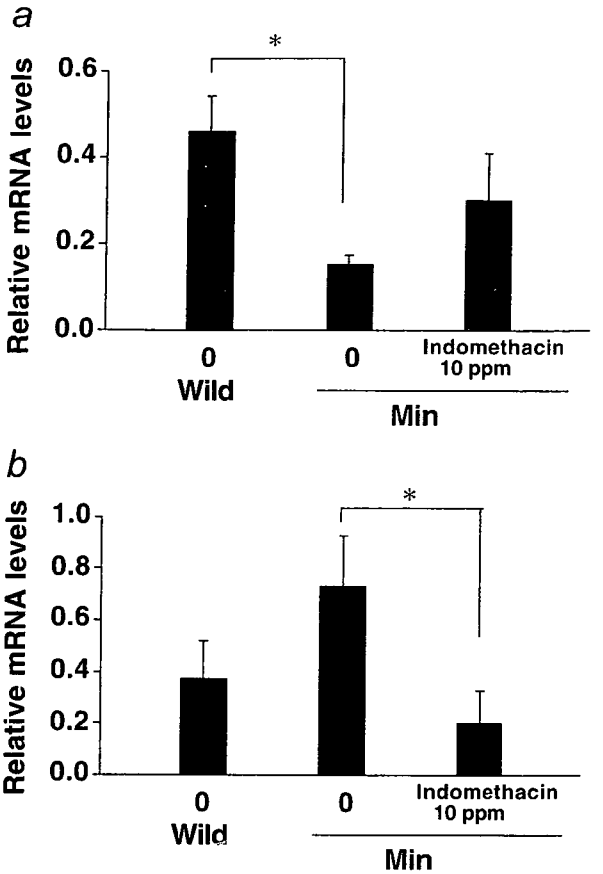

FIGURE 2 – Changes of LPL and cytokine mRNA levels in Min mice. LPL mRNA expression levels in the livers of female Min and wild-type mice treated with indomethacin at 10 ppm in the diet for 14 weeks are shown (*a*). Hepatic mRNA levels for TNFα are shown in female Min and the wild-type mice (*b*). Data are means ($n = 5$); *bars*, SE. $*p < 0.05$.

downregulated as compared to those in wild-type mice (Fig. 2). Treatment with indomethacin at 10 ppm in diet for 14 weeks from 6 weeks of age slightly increased LPL mRNA levels 2-fold in Min mice, although the value is not statistically significant (Fig. 2*a*).

As this elevation could not fully explain the improvement in hyperlipidemia, changes in hepatic gene expression were analyzed comprehensively using the 12,488 probe set on GeneChip Murine Genome U74A®. Three liver samples each from 0 and 10 ppm indomethacin-treated groups were employed for this purpose. Fourteen weeks administration of 10 ppm indomethacin in Min mice caused significant changes of gene expression levels with 277 probes compared to the untreated control group. Nineteen genes were significantly ($p < 0.05$) upregulated 2-fold or more, and 18 genes were downregulated by 1/2 or more. Among the total of 37 genes, 8 were related to lipid metabolism (Table II). Quantitative real-time PCR confirmed the results for expression levels observed with the array (Table II). Four genes (SPOT14, ATP citrate lyase, lanosterol synthase and retinol dehydrogenase 11) were obviously increased and the remaining 4 (glycerol kinase, HMG-CoA synthase 2, monoglyceride lipase and acyl-CoA thioesterase 1) were obviously decreased.

TABLE II – LIPID METABOLISM-RELATED GENES IN THE LIVER OF MIN MICE

Genes	Fold changes[1]	
	Microarray	Real-time PCR
SPOT14	5.4	3.0
ATP citrate lyase	4.9	2.8
Lanosterol synthase	3.1	2.2
Retinol dehydrogenase 11	3.1	2.3
Glycerol kinase	0.5	0.4
HMG-CoA synthase 2	0.5	0.3
Monoglyceride lipase	0.5	0.4
Acyl-CoA thioesterase 1	0.2	0.3

[1]Indomethacin-treated/untreated.

Hepatic mRNA levels of TNFα

Lipid metabolism is closely linked to inflammatory signaling pathways.[22] Thus, mRNA levels for TNFα in the liver of Min mice were examined by real-time PCR. As shown in Figure 2, hepatic mRNA levels of TNFα were higher in Min mice when compared with wild-type mice (2-fold). Indomethacin treatment suppressed the high TNFα mRNA expression in Min mice to almost the wild-type value (Fig. 2*b*).

Discussion

The present study provided clear evidence that indomethacin can suppress hyperlipidemia, along with reduction of intestinal polyps in Min mice as previously reported.[11] Thus significant decrease in serum levels of triglycerides, total cholesterol, VLDL-C, and LDL-C was observed, along with increase in HDL-C. The reduction (90%) in serum triglycerides in Min mice with 10 ppm indomethacin treatment was higher than with 400 ppm pioglitazone, a PPARγ agonist, observed earlier (83%) in our previous study.[14] On the other hand, the PPARγ agonistic activity of indomethacin is reported to be 50 times weaker than that of the well-established PPARγ agonist, troglitazone.[23] Our previous and present results lead us to hypothesize that functions other than the weak PPARγ agonistic activity of indomethacin are responsible for its strong lipid-lowering effects.

The DNA array analysis revealed HMG-CoA synthase 2 and glycerol kinase to be downregulated, and lanosterol synthase, ATP

citrate lyase, SPOT14 and retinol dehydrogenase 11 to be upregulated. Interestingly, these genes involved in lipid metabolism are all regulated by sterol regulatory element binding protein (SREBP)-1c at the transcriptional level, although there were no differences in the expression levels of acetyl-CoA carboxylase, one of the lipogenic genes transcriptionally regulated by SREBP-1c.[20,24–26] Moreover, LPL is another target gene of SREBP-1c.[27] SREBPs (SREBP-1c, SREBP-1a and SREBP-2) are membrane-bound transcription factors involved in lipid synthetic gene regulation in the liver.[19,24] Among SREBPs, hepatic SREBP-1c is induced by excess energy intake, and plays a role in fatty acid synthesis and insulin related glycogenesis.[20] Notably, it is also reported that SREBPs may play a role in carcinogenesis,[28] with upregulation observed in human colorectal carcinomas, prostate cancers, hepatocellular carcinomas and primary breast cancers.[28–31]

Recently, activation of glycerol kinase was reported to accelerate triglyceride synthesis in the aquaporin 7-knockout mouse[32] and in the present study we found a decrease of glycerol kinase mRNA levels in the livers of indomethacin-treated Min mice. Thus, downregulation of glycerol kinase, a SREBP-1c and PPARα target gene,[26,33] may cause decrease serum triglyceride levels. Moreover, indomethacin suppression of HMG-CoA synthase, the enzyme preceding HMG-CoA reductase which catalyzes the first committed step in the mevalonate pathway,[25] could also be essentially involved in serum cholesterol reduction in Min mice. Meanwhile, mRNA levels for ATP citrate lyase and SPOT14 were reported to be markedly activated in the livers of mice during fasting-refeeding treatment,[34] suggesting that the increase in ATP citrate lyase and SPOT14 mRNA levels in indomethacin-treated Min mice in the present study was due to physiological response against serum lipid reduction.

It has been reported that serum triglyceride levels are increased by inflammatory cytokines and adipocytokines, such as TNFα and interleukin (IL)-6.[35–37] These cytokines rapidly induce de novo free fatty acid synthesis and hepatic triglyceride synthesis.[38] TNFα also induces a mature form of SREBP-1 in human hepatocytes and promotes transcription of SREBP-1 target genes.[39] In addition, TNFα and IL-6 play important roles in obesity, type 2 diabetes, and hemorrhage.[40,41] In the present study, liver TNFα mRNA levels were increased in Min mice and downregulated by indomethacin. Furthermore, we have reported previously that pioglitazone, which increases insulin sensitivity, and an LPL inducer, NO-1886, improve hypertriglyceridemia by inducing LPL mRNA and also suppressing intestinal polyp formation in *Apc*-deficient mice.[13–15] We can therefore speculate that indomethacin impacts on intestinal tumor formation together with the hyperlipidemic state by regulating LPL and other lipid metabolic and inflammatory pathways. Colon carcinogenesis is highly related to expression of lipogenic enzymes or adipocytokine production. The expression levels of adipocytokines such as IL-6, leptin and adiponectin in Min mice treated with or without indomethacin are now under investigation in our laboratory.

In conclusion, this is the first report to show that indomethacincan improve not only intestinal polyposis but also hyperlipidemia in Min mice, with suggestive influence of SREBP-1c activation and TNFα expression levels. Indomethacin has long been clinically used as an antiinflammatory drug with indications for reducing intestinal polyp formation and colon carcinogenesis.[7,11] On the basis of the present study, possible inhibitory effects of indomethacin on hyperlipidemia should also be linked to polyp decrement. Further testing in clinical studies is clearly warranted.

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A specific inducible nitric oxide synthase inhibitor, ONO-1714 attenuates inflammation-related large bowel carcinogenesis in male $Apc^{Min/+}$ mice

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It is generally assumed that inflammation influences carcinogenesis. We previously reported that dextran sodium sulfate (DSS) strongly enhances colon carcinogenesis in the $Apc^{Min/+}$ mice and the over-expression of inducible nitric oxide synthase (iNOS) contributes to this enhancement. In the current study, we investigated the effect of a selective iNOS inhibitor, ONO-1714 on colitis-related colon carcinogenesis in the $Apc^{Min/+}$ mouse treated with DSS. Male C57BL/6J $Apc^{Min/+}$ and $Apc^{+/+}$ mice were exposed to 1% DSS in their drinking water for 7 days. ONO-1714 was given to the mice at a dose level of 50 or 100 ppm in diet for 5 weeks (during the administration of DSS). The tumor inhibitory effects by ONO-1714 were assessed at week 5 by counting the incidence and multiplicity of colonic neoplasms. Additionally, we assessed serum lipid levels and colonic mRNA expression for cyclooxygenase (COX)-2, iNOS, tumor necrosis factor (TNF)-α and interleukin (IL)-1β. Feeding with ONO-1714 significantly inhibited the occurrence of colonic adenocarcinoma in a dose-dependent manner in the $Apc^{Min/+}$ mice. In addition, the treatment with ONO-1714 significantly lowered the serum triglyceride levels and mRNA expression levels of COX-2, TNFα and IL-1β of colonic mucosa in the DSS-treated $Apc^{Min/+}$ mice. Neither ONO-1714 nor DSS affected the colonic pathology in the $Apc^{+/+}$ mice. Our findings may suggest that ONO-1714 could therefore serve as an effective agent for suppression of colitis-related colon cancer development in the $Apc^{Min/+}$ mice.

Key words: colitis-related carcinogenesis; chemoprevention; dextran sodium sulfate; iNOS; $Apc^{Min/+}$ mice

The link between carcinogenesis and chronic inflammation has been recognized for certain types of cancer, including colorectal cancer (CRC).[1] CRC is one of the known serious complications of inflammatory bowel disease (IBD), including ulcerative colitis (UC).[1,2] For understanding the pathogenesis of IBD and IBD-related CRC, we and others have been studying the colitis-associated carcinogenesis process using the dextran sodium sulfate (DSS) model in mice.[3–7] In our previous studies, inflammation/inflammatory stimuli induced by DSS treatment after the initiation with a low-dose of colonic carcinogen is effective for the rapid induction of colonic neoplasms that possess β-*catenin* gene mutations in ICR mice.[4,5] Furthermore, we recently reported that DSS treatment results in intestinal mucosa inflammation and numerous colorectal neoplasms in $Apc^{Min/+}$ mice, which demonstrates a germline mutation in the adenomatous polyposis coli (APC) gene.[8] Cooper *et al.*[7] also found a relationship between the severity of DSS-induced inflammation and colorectal carcinogenesis in the $Apc^{Min/+}$ mice. Therefore, DSS models with or without carcinogen can be useful for investigating the IBD-associated colorectal carcinogenesis.

Nitric oxide (NO) is a mediator of physiological processes in the gastrointestinal tract, including mucosal protection, the regulation of blood flow and the regulation of motility.[9] On the other hand, the overproduction of NO contributes to tissue damage, colon tumor growth and DNA deamination.[1,10] Increased NO production as well as the expression of inducible nitric oxide synthase (iNOS) in intestinal mucosa is known to be associated with the disease activity of IBD.[11] In colon carcinogenesis, an increased expression of iNOS was up-regulated in human colon adenomas and adenocarcinomas,[1,10] and azoxymethane (AOM)-induced rat large intestinal aberrant crypt foci (ACF), and tumors as well.[12] We also observed that the immunohistochemical expression of iNOS increased in inflamed colonic mucosa and colonic adenocarcinoma in the mice that received AOM and/or DSS.[3,8] In addition, the oxidative/nitrosative stress caused by DSS exposure contributes to the development of a high incidence of colonic adenocarcinomas in mice.[13] Therefore, NO and iNOS may play a certain role in the experimental inflammation-related colon carcinogenesis and the development of human UC-associated cancer.[1]

According to these data, treatment with several selective iNOS inhibitors may serve as a novel experimental approach to colitis and/or colitis-associated carcinogenesis.[10] Among these iNOS inhibitors, ONO-1714, being 10-fold more selective for human iNOS than for human endothelial NOS, is very potent with an ID_{50} value of 0.010 mg/kg s.c. and lowly toxic with a maximum tolerated dose of 30 mg/kg i.v. in mice.[14,15] These biological natures of ONO-1714 and its effectiveness even when orally administered[16] thus led us to investigate the modifying effects of ONO-1714 on colon carcinogenesis in the $Apc^{Min/+}$ mice receiving DSS.

Material and methods

Animals, chemicals and diets

Male C57BL/6J $Apc^{Min/+}$ and $Apc^{+/+}$ were purchased from the Jackson Laboratory (Bar Harbor, ME) at 5 weeks of age and they were genotyped by the method reported previously.[17] They were maintained at the Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines, quarantined for the first 7 days, and then randomized by body weight into ex-

Abbreviations: Alb, albumin; A/G, albumin:globlin ratio; ACF, aberrant crypt foci; ALT, alanine aminotransferase; ALP, alkaline phosphatase; APC, adenomatous polyposis coli; AST, aspartate aminotransferase; AOM, azoxymethane; BUN, blood urea nitrogen; CRC, colorectal cancer; COX, cyclooxygenase; DSS, dextran sodium sulfate; eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; Glu, glucose; H&E, hematoxylin and eosin; IBD, inflammatory bowel disease; iNOS, inducible nitric oxide synthase; IL, interleukin; LDH, lactate dehydrogenase; nNOS, neural nitric oxide synthase; NO, nitric oxide; RT-PCR, reverse transcriptase-polymerase chain reaction; T-Bil, total bilirubin; T-Cho, total cholesterol; TP, total protein; TG, triglyceride; TNF, tumor necrosis factor; UC, ulcerative colitis.

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TABLE I – BODY WEIGHTS, LIVER WEIGHTS, RELATIVE LIVER WEIGHTS AND LENGTHS OF LARGE BOWEL

Group no.	Treatment (no. of mice)	Body wt. (g)	Liver wt. (g)	Relative liver wt. (g/100 g body wt.)	Length of large bowel (cm)
$Apc^{Min/+}$	1% DSS (12)	20.4 ± 4.4[1]	1.01 ± 0.24	4.95 ± 0.52	8.25 ± 0.42
	1% DSS + 50 ppm ONO-1714 (10)	20.8 ± 4.2	0.88 ± 0.27	4.27 ± 1.09	8.79 ± 0.63[2]
	1% DSS + 100 ppm ONO-1714 (10)	19.2 ± 3.1	0.84 ± 0.18	4.36 ± 0.58	9.22 ± 0.34[3]
$Apc^{+/+}$	1% DSS (5)	26.8 ± 2.1	1.19 ± 0.36	4.48 ± 1.45	10.06 ± 0.29[3]
	1% DSS + 50 ppm ONO-1714 (5)	24.0 ± 2.1	1.09 ± 0.30	4.49 ± 1.01	10.25 ± 0.31
	1% DSS + 100 ppm ONO-1714 (5)	23.5 ± 2.1	1.19 ± 0.25	5.09 ± 1.15	10.16 ± 0.46

[1]Mean ± SD.–[2,3]Significantly different from the "1% DSS" group of $Apc^{Min/+}$ mice by Tukey's multiple comparison post test ($^2p < 0.05$ and $^3p < 0.001$).

perimental and control groups. All animals were housed in plastic cages (4 or 5 mice/cage) with free access to drinking water and a powdered basal diet AIN-76A (Oriental Yeast, Tokyo, Japan), under controlled conditions of humidity [(50 ± 10)%], light (12/12 hr light/dark cycle) and temperature [(23 ± 2)°C]. DSS with a molecular weight of 36,000–50,000 (Cat No. 160110) was purchased from MP Biochemicals, LLC (Aurora, OH, USA). DSS was dissolved in tap water at a concentration of 2% (w/v). ONO-1714 was chemically synthesized at Ono Pharmaceutical (Osaka, Japan). The experimental diets were prepared by mixing ONO-1714 (50 and 100 ppm) with modified AIN-76A diet every week.

Experimental procedures

Forty-seven male $Apc^{Min/+}$ and 30 male $Apc^{+/+}$ mice were divided into experimental and control groups as shown in the Tables. The animals of the experimental groups were administered 1% (w/v) DSS in drinking water for 1 week from 6 weeks of age. They were also given a basal diet (modified AIN-76A) or a diet containing ONO-1714 at a dose of 50 or 100 ppm for 5 weeks from 6 weeks of age. The doses were determined based on the results of previous studies.[16,18] Food and water were available *ad libitum*. The animals were observed daily for clinical signs and mortality. The body weights and food consumption were measured weekly. At sacrifice (week 5), the animals were anesthetized with ether, and blood samples were collected from the abdominal aorta. They were starved overnight prior to blood collection. Clinical chemistry measured included triglyceride (TG), total cholesterol (T-Cho), glucose (Glu), total protein (TP), albumin (Alb), albumin:globlin ratio (A/G), total bilirubin (T-Bil), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), creatinine and blood urea nitrogen (BUN). To assess relative quantification of gene expression in colonic mucosa, samples of colonic mucosa of mice ($n = 5$ per group) were washed with phosphate buffer saline, quickly frozen in liquid nitrogen, and stored at −80°C. The intestinal tract of the remaining mice was removed, filled with 10% phosphate-buffed formalin, and then divided into 4 sections; the colon and 3 segments of small intestine; the proximal (~4 cm in length from the pylorus ring of stomach), and the middle and distal halves of the remainder. These segments were opened longitudinally and fixed flat between sheets of filter paper in 10% phosphate-buffered formalin. Polyp numbers and sizes, and their distributions in the small intestine were determined under a dissecting microscope Nikon SMZ1000 (Nikon, Tokyo, Japan), as described.[19] A histological examination was performed on paraffin-embedded sections after hematoxylin and eosin (H&E) staining. Intestinal neoplasms were diagnosed according to the description by Ward.[20]

Scoring of inflammation in the intestinal mucosa

Mucosal inflammation with or without ulceration in the entire intestine was analyzed on H&E-stained sections. Small and large intestinal inflammation was graded according to the following morphological criteria described by Cooper *et al.*[21]: Grade 0, normal appearance; Grade 1, shortening and loss of the basal one-third of the actual crypts with mild inflammation in the mucosa; Grade 2, loss of the basal two-thirds of the crypts with moderate inflammation in the mucosa; Grade 3, loss of the entire crypts with severe inflammation in the mucosa and submucosa, but with retainment of the surface epithelium and Grade 4, presence of mucosal ulcer with severe inflammation (neutrophil, lymphocyte and plasma cell infiltration) in the mucosa, submucosa, muscularis propria and/or subserosa. The scoring was made based on the entire intestine with or without proliferative lesions and expressed as a mean average score/mouse.

Immunohistochemistry

Immunohistochemistry for COX-2 and iNOS was performed on 4-μm-thick paraffin-embedded sections from colons of $Apc^{Min/+}$ mice in each group as previously described.[3,8] As primary antibodies, anti-COX-2 mouse monoclonal antibody (1:200 dilution, Transduction Laboratories, Lexington, KY) and anti-iNOS mouse monoclonal antibody (1:250 dilution, Transduction Laboratories) were used. To reduce the nonspecific staining of mouse tissue by the mouse antibodies, a Mouse On Mouse IgG blocking reagent (Vector Laboratories, Burlingame, CA) was applied. Horseradish peroxidase activity was visualized by treatment with H_2O_2 and 3,3′-diaminobenzidine for 5 min. In the last step, the sections were weakly counterstained with Mayer's hematoxylin (Merck, Tokyo, Japan). For each case, negative controls were performed on serial sections. In the control sections, incubation with the primary antibodies was omitted.

RNA extraction and synthesis of cDNA

The extraction of total RNA from frozen colonic mucosa was done using the RNeasy® Mini Kit (Qiagen, CA) following the manufacturer's directions. The concentration of total RNA was measured with a spectrophotometer and the A_{260}/A_{280} ratio of RNA was 1.8–2.0. cDNA was synthesized by oligo (dT)20 primer and SuperScript™ III First-Strand Synthesis System for reverse transcriptase-polymerase chain reaction (RT-PCR) (Invitrogen, Life Technologies, Paisley, UK) according to the manufacturer's protocol, and then it was stored at −30°C until analyzed.

Relative quantification of gene expression by LightCycler RT-PCR

All real-time experiments were carried out with a LightCycler® FastStart DNA Master HybProbe kit (Roche, Mannheim, Germany). Oligonucleotide primers and hybridization probes are purchased from Nihon Gene Research Lab's (Sendai, Japan). PCR was performed with specific primers for mouse cyclooxygenase (COX)-2 (forward primer; 5′-CCATCTGTTCTCCTCAATAC-3′, reverse primer; 5′-TTTGGTAGGCTG TGGAT-3′), iNOS (forward primer; 5′-GCAAACCCAAGGTCTACGTT-3′, reverse primer; 5′-GGAAAAGACTGCACCGAAGA-3′), interleukin (IL)-1β (forward primer; 5′-CTGTGGCAGCTACCTGTGTC-3′, reverse primer; 5′-GTTCATCTCGGAGCC TGTAG-3′), tumor necrosis factor (TNF)-α (forward primer; 5′-CCACGTCGTAGC AAACCAC-3′, reverse primer; 5′-TGGGTGAGGAGCACGTAGT-3′) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (forward primer; 5′-TGAACGGGAAGCTCACTGG-3′, reverse primer; 5′-TCCACCACCCTGTTG CTGTA-3′). PCR reactions contained 15 μl of master mix and 5 μl of template cDNA. The final reaction mix-

ture contained: for GAPDH, iNOS, IL-1β and TNF-α, 3 mM $MgCl_2$, 0.5 μM of the primers (Forward and Reverse), 0.2 μM of the Fluorescein probe, 0.4 μM of the and LCRed probe and 1X LightCycler (FastStart DNA Master HybProbe); and for cyclooxygenase (COX)-2, 3 mM $MgCl_2$, 0.2 μM of the primers (Forward and Reverse), 0.2 μM of the probes (Fluorescein and LCRed) and 1X LightCycler® FastStart DNA Master HybProbe based on the manufacturer's recommendations. The contents were placed in a glass capillary, capped, briefly centrifuged and placed in the LightCycler. The denaturing condition for all genes was 1 cycle at 95°C for 10 min. The amplification conditions were as follows: GAPDH, 40 cycles of 95°C for 10 sec, 60°C for 15 sec and 72°C for 9 sec; iNOS, 40 cycles of 95°C for 10 sec, 62°C for 15 sec and 72°C for 9 sec; IL-1β and TNF-α, 40 cycles of 95°C for 10 sec, 61°C for 15 sec and 72°C for 7 sec; and COX-2, 45 cycles of 95°C for 10 sec, 62°C for 15 sec and 72°C for 7 sec. The amplification protocol was followed by a cooling period of 1 cycle at 40°C for 30 sec. All data collection were performed during extension and the data were monitored through the F2/1 channel of the instrument. Data analyses were carried out using the second derivative maximum method of the LightCycler software program.

Statistical analysis

All measurements were compared by Tukey's multiple comparison post test, Bonferroni's multiple comparison post test or Fisher's exact probability test. Differences were considered to be statistically significant at $p < 0.05$.

Results

General observation

During the study, animals tolerated the oral administration of DSS and ONO-1714, and no clinical signs of toxicity were present in any groups. The intake of DSS and food consumption (g/day/mice) did not significantly differ among the groups (data not shown). The body weights, liver weights, relative liver weights and lengths of large bowel at the end of the study are given in Table I. The mean body weights, liver weights and relative liver weights did not significantly differ among the groups. The mean length of large bowel in the groups of $Apc^{Min/+}$ mice treated with ONO-1714 at 50 and 100 ppm doses were significantly longer than that of the DSS alone group ($p < 0.05$ and $p < 0.01$, respectively). Histologically, there were no pathological alterations suggesting the toxicity of ONO-1714 in the liver, kidneys, lung and heart in mice.

Pathological findings

Macroscopically, nodular and polypoid colonic tumors were observed in the cecum and colon of $Apc^{Min/+}$ mice that received DSS, but not in the $Apc^{+/+}$ mice. Histopathologically, colonic proliferative lesions, including dysplastic crypts, adenomas and adenocarcinomas were found in the cecum and colon of $Apc^{Min/+}$ mice treated with DSS. The incidences and multiplicities of large bowel neoplasma are shown in Table II. The incidences of large bowel tumors in the $Apc^{Min/+}$ mice did not significantly differ among the DSS alone, DSS + 50 ppm ONO-1714 and DSS + 100 ppm ONO-1714 groups. However, the multiplicities of large bowel tumors in the groups of $Apc^{Min/+}$ mice that received DSS and ONO-1714 at dose levels of 50 and 100 ppm were significantly lower than that of the DSS alone groups ($p < 0.05$ and $p < 0.05$, respectively). Although the multiplicities of adenomas did not significantly differ among the groups, the multiplicities of the adenocarcinomas significantly decreased by feeding with ONO-1714 to 49% (50 ppm, $p < 0.001$) and 58% (100 ppm, $p < 0.001$) of the value of DSS alone group. The number of small intestinal polyps (adenomas) of the DSS and ONO-1714-treated groups were lower than that of the DSS alone group, but the differences among the groups were not statistically significant (Table III).

TABLE II - EFFECT OF ONO-1714 ON THE DEVELOPMENT OF LARGE BOWEL TUMORS IN THE I $Apc^{Min/+}$ AND $Apc^{+/+}$ MICE THAT RECEIVED DSS OR DSS + ONO-1714

Geno-type	Treatment (no. of mice)	Colon			Cecum			Large bowel (total)		
		AD[1] (incidence)	ADC[2] (incidence)	Total (incidence)	AD (incidence)	ADC (incidence)	Total (incidence)	AD (incidence)	ADC (incidence)	Total(incidence)
$Apc^{Min/+}$	1% DSS (12)	3.25 ± 2.30[3] (100%)	4.58 ± 2.43 (100%)	7.83 ± 4.32 (100%)	5.00 ± 2.80 (100%)	11.50 ± 5.60 (100%)	16.50 ± 6.97 (100%)	8.25 ± 3.65 (100%)	16.08 ± 5.76 (100%)	24.33 ± 8.68 (100%)
	1% DSS + 50 ppm ONO-1714 (10)	3.10 ± 1.52 (100%)	1.40 ± 0.84[4] (90%)	4.50 ± 1.96 (100%)	5.00 ± 1.83 (100%)	6.80 ± 2.57[5] (100%)	11.80 ± 3.29 (100%)	8.10 ± 2.77 (100%)	8.20 ± 3.12[3] (100%)	16.30 ± 4.19[5] (100%)
	1% DSS + 100 ppm ONO-1714 (10)	3.70 ± 1.42 (90%)	1.40 ± 0.97[4] (100%)	5.10 ± 1.97 (100%)	5.70 ± 2.16 (100%)	5.30 ± 2.06[5] (100%)	11.00 ± 3.83 (100%)	9.40 ± 2.76 (100%)	6.70 ± 2.67[3] (100%)	16.10 ± 5.02[5] (100%)
$Apc^{+/+}$	1% DSS (5)	0	0	0	0	0	0	0	0	0
	1% DSS DSS + 50 ppm ONO-1714 (5)	0	0	0	0	0	0	0	0	0
	1% DSS DSS + 50 ppm ONO-1714 (5)	0	0	0	0	0	0	0	0	0

[1]AD, adenoma.–[2]ADC, adenocarcinoma.–[3]Mean ± SD.[4,5]Significantly different from the "1% DSS" group of $Apc^{Min/+}$ mice by Tukey's multiple comparison post test ([4]$p < 0.001$ and [5]$p < 0.05$).