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Improvement of hyperlipidemia by indomethacin in Min mice

Naoko Niho[1], Michihiro Mutoh[1], Masami Komiya[1], Tsutomu Ohta[2], Takashi Sugimura[1] and Keiji Wakabayashi[1]*

[1]*Cancer Prevention Basic Research Project, National Cancer Center Research Institute, Tokyo, Japan*
[2]*Center for Medical Genomics, National Cancer Center Research Institute, Tokyo, Japan*

***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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***Correspondence to:** Cancer Prevention Basic Research Project, National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan. Fax: +81-3-3543-9305.
E-mail: kwakabay@gan2.res.ncc.go.jp

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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			Colon	Total
		Proximal	Middle	Distal		
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

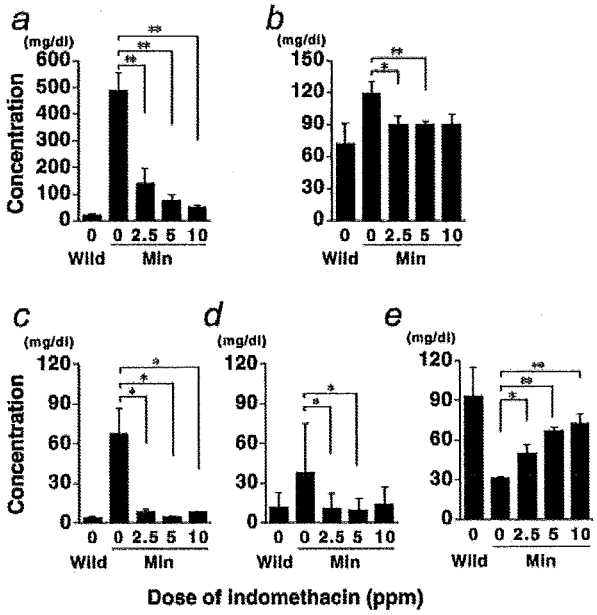

FIGURE 1 – Suppression of serum lipid levels in Min mice by indomethacin. Values for serum levels of triglyceride (*a*), total cholesterol (*b*), VLDL-C (*c*), LDL-C (*d*), and HDL-C (*e*) in female Min mice given basal diet or diet containing indomethacin at doses of 2.5, 5 and 10 ppm for 14 weeks and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

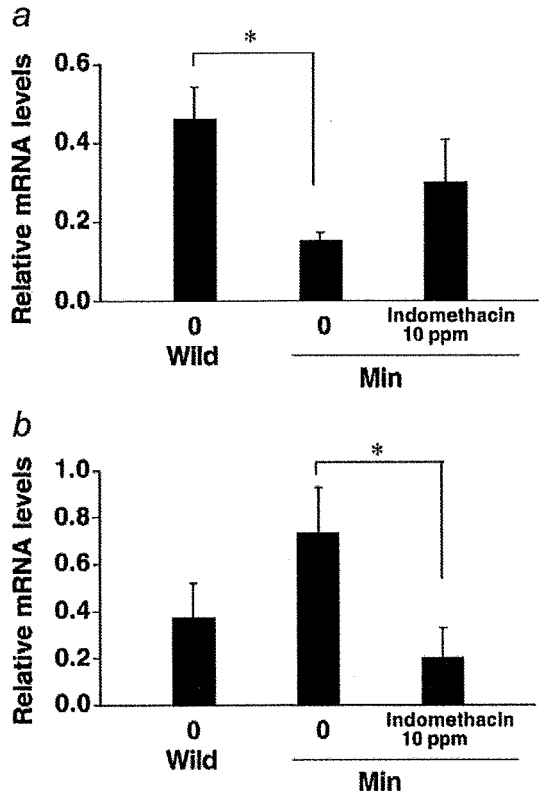

FIGURE 2 – Changes of LPL and cytokine mRNA levels in Min mice. LPL mRNA expression levels in the livers of female Min and wild-type mice treated with indomethacin at 10 ppm in the diet for 14 weeks are shown (*a*). Hepatic mRNA levels for TNFα are shown in female Min and the wild-type mice (*b*). Data 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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Enhanced thyroid carcinogenicity of *N*-nitrosobis(2-oxopropyl)amine in Otsuka Long-Evans Tokushima Fatty rats, a model of type II diabetes mellitus

Katsuhisa Sakano, Mami Takahashi, Michihiro Mutoh, Naoko Niho, Masami Komiya, Hidetaka Sato[1], Takuji Tanaka[2], Takashi Sugimura and Keiji Wakabayashi*

Cancer Prevention Basic Research Project, National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan, [1]Japan Food Research Laboratories, Bunkyo 2-3, Chitose-shi, Hokkaido 066-0052, Japan and [2]Department of Oncologic Pathology, Kanazawa Medical University, 1-1 Daigaku, Uchida, Ishikawa 920-0293, Japan

*To whom correspondence should be addressed. Tel: +81 3 3542 2511 ext. 4350; Fax: +81 3 3543 9305
Email: kwakabay@gan2.res.ncc.go.jp

Epidemiologic data suggest that diabetes mellitus type II is a risk factor for several types of cancer, including pancreatic, liver, colon and thyroid cancers. In the present study, effects of diabetes/hyperlipidemia on *N*-nitrosobis(2-oxopropyl)amine (BOP)-induced cancer development were examined in Otsuka Long-Evans Tokushima Fatty (OLETF) rats, model animals for non-insulin-dependent diabetes mellitus and Long-Evans Tokushima Otsuka (LETO) rats, appropriate controls. Males of both strains were given four subcutaneous injections of BOP (10 mg/kg body wt) or saline on alternative days, starting at 5 weeks of age. BOP induced tumors in a variety of tissues, including the thyroid gland, colon, kidney, liver and lung. The highest yields were noted for thyroid tumors, the incidence ($P = 0.0182$) and multiplicity ($P < 0.001$) of BOP-induced thyroid cancers with marked fibrosis being significantly higher in OLETF than in LETO rats. Interestingly, anaplastic thyroid carcinomas were observed limited to the BOP-treated OLETF rats. Additionally, a greater incidence and frequency of aberrant crypt foci, putative precursor lesions for colon tumors, was observed in the BOP-treated OLETF group. However, BOP was ineffective at inducing pancreatic ductal tumors. No thyroid, liver, lung or colon tumors were found in the OLETF and LETO rats receiving the vehicle. Significant increases in serum levels of insulin, glucose, phospholipids, triglycerides and total cholesterol were detected in the OLETF rats compared with the LETO rats, regardless of the treatment. Our results indicate that diabetic/hyperlipidemic state can enhance BOP-induced carcinogenesis of the thyroid gland and to a lesser extent the colon in OLETF rats.

Introduction

Diabetes mellitus is a group of common metabolic diseases that feature hyperglycemia in common and are classified into the two broad categories, type I and type II, on the basis of the underlying pathogenic processes. Type I diabetes, which accounts for about 5–10% of patients, is a state of absolute deficiency of insulin caused mainly by autoimmune destruction of pancreatic β cells. Type II diabetes accounting for >90% of cases is characterized by high insulin resistance in fat and muscle tissues and leads to an inadequate, compensatory increased production of insulin. Decompensation of β cells and low absolute insulin concentrations eventually occur, but only in later stages of the disease. Type II diabetes is a major health problem in developed countries, where it affects ~7% of adults and ~15% of people older than 60 years. Although the major complication of diabetes mellitus is microangiopathy, diabetes mellitus type II is known to be associated with various neoplasms and is recognized as a risk factor for several types of cancer, including pancreatic, liver, colon and thyroid cancers (1–3). In fact, the risk of colon cancer appears to be elevated by a high-fat diet, and epidemiological studies have shown a clear association with hypertriglycemia and hypercholesteronemia (4,5). Epidemiological findings have also shown that fat intake and obesity might increase pancreatic cancer risk and that diabetes mellitus might be related with thyroid cancer (3,6,7).

The Otsuka Long-Evans Tokushima Fatty (OLETF) strain of rats spontaneously develops hyperglycemia, hyperinsulinemia, insulin resistance and mild obesity and has been well studied as an animal model for type II diabetes mellitus (8–10). OLETF rats show late onset of hyperglycemia (after 20 weeks of age), mild course of diabetes, and conversion to insulin-dependent diabetes after 40 weeks of age (8–10). It has been reported that both males and females develop atypical hyperplasia of the choledochopancreatic duct by 15 weeks of age (11), frequently accompanied by papillary growth with cellular atypia. However, no progression of the lesion to malignant tumors has been observed in older OLETF rats.

N-Nitrosobis (2-oxopropyl) amine (BOP) is a potent pancreatic carcinogen, being able to induce a high incidence of pancreatic ductal neoplasms, both adenomas and adenocarcinomas, in Syrian hamsters (12,13). In contrast, in rats, BOP induces tumors mainly in the thyroid, liver and lungs (14–16). In the present study, we examined the carcinogenic potency of BOP in the OLETF and Long-Evans Tokushima Otsuka (LETO) rats, appropriate controls, to determine whether diabetic and/or hyperlipidemic conditions influence carcinogenesis by BOP.

Materials and methods

Animals and chemicals

Male OLETF and counterpart control LETO rats were kindly supplied by the Tokushima Research Institute, Otsuka Pharmaceutical Co, Ltd (Tokukshima, Japan) when 4 weeks old and weighing ~100 g. They were acclimated to laboratory conditions for a week, housed one per plastic cage with sterilized softwood chips as bedding in a barrier-sustained animal room, air-conditioned at 24 ± 2°C and 55% humidity, on a 12 h light/dark cycle. CE-2 (CLEA Japan, Shizuoka, Japan) was employed as a standard basal diet. Food and water were available *ad libitum*. Body weights and food consumption were measured weekly during the study. BOP was obtained from Nacalai Tesque (Kyoto, Japan). The experimental protocols were approved by the Institutional Ethics Review Committee for Animal Experimentation.

Carcinogenicity study

Groups of 25 OLETF and LETO rats at 5 weeks of age were each given four subcutaneous injections of BOP on days 1, 3, 5 and 7 at a dose of 10 mg/kg body wt, and additional groups of 25 animals each received saline alone as vehicle controls. At the final sacrifice time point at weeks 62–64 (67–69 weeks of age), all surviving animals were anesthetized with diethyl ether, and blood samples were collected from the abdominal aorta for measurement of various serum parameters, including insulin, glucose, triglyceride, total cholesterol, phospholipids, free fatty acids, tetraiodothyronine (T4), triiodothyronine (T3) and thyroid-stimulating hormone (TSH), conducted by SRL, Inc. (Tokyo, Japan). At autopsy, the pancreas, thyroid, heart, lung, kidneys, liver, spleen, testis, stomach, intestine (small intestine, cecum and colon) and bile duct were carefully examined macroscopically. Each pancreas and colon were carefully dissected from surrounding tissue and fixed in 10% phosphate-buffered formalin (pH 7.4) after spreading on filter paper. The other organs were also fixed in 10% phosphate-buffered formalin (pH 7.4) and routinely processed for embedding in paraffin. Sections were stained with hematoxylin and eosin for assessment of histopathological features.

Histopathological examination of thyroid tumors and scoring of fibrosis

Thyroid neoplasms were histopatholgically diagnosed according to the criteria described by Kawaoi *et al.* (17). Fibrosis in the thyroid glands was assessed as percent areas of thyroid sections. The image analysis software National

Abbreviations: ACF, aberrant crypt foci; BOP, *N*-nitrosobis(2-oxopropyl)-amine; LETO, Long-Evans Tokushima Otsuka; OLETF, Otsuka Long-Evans Tokushima Fatty; TSH, thyroid-stimulating hormone; T3, triiodothyronine; T4, tetraiodothyronine.

Institutes of Health Image v.1.63 was used for calculation and the following grading system was applied to quantify the degree of fibrosis: grade 0 (<5% fibrosis), grade 1 (<6–20% fibrosis), grade 2 (21–35% fibrosis), grade 3 (36–50% fibrosis), grade 4 (51–65%) and grade 5 (>66%).

Determination of aberrant crypt foci

All colons were carefully removed, flushed with saline, slit open longitudinally from the cecum to the anus, placed between filter papers and fixed in 10% neutral buffered formalin for 24 h. They were then stained with 0.2% methylene blue in saline and placed, mucosal side up, on a microscope slide and examined under a microscope. Aberrant crypt foci (ACF) were recorded according to standard procedures used routinely in our laboratory (18).

Statistical analysis

The significance of differences in the incidences of tumors and other lesions was analyzed by the Fisher's exact probability test. Variation in other data was evaluated by the Student's unpaired *t*-test, one-way analysis of variance and *post hoc* (Tukey) multiple comparison test or one-way analysis of variance with a Bonferroni correction for multiple comparisons. A P value of <0.05 was regarded as significant.

Results

General observation

In the BOP-treated OLETF group, 15 rats were killed or died between weeks 42 and 58. At week 42, one rat was killed because of a large tumor mass developing in the lower abdomen. The mass was histopathologically diagnosed as granulomatous prostatitis and seminal vesiculitis. The first tumor that developed in the thyroid of an OLETF rat treated with BOP was observed at week 44, and therefore animals that survived beyond this time point were counted in the effective numbers. In the saline-treated OLETF group, six rats were killed or died at weeks 37–58. One rat of this group developed an inflammatory granuloma in the forestomach. In the BOP-treated LETO group, one rat was killed at week 32 because of dyspnea, but had not developed any tumors. In the saline-treated LETO group, one rat that had an inflammatory granuloma in the forestomach was killed at week 48. The final survival rates at weeks 62–64 were 76% (19/25 rats) in the saline-treated OLETF group, 96% (24/25 rats) in the saline-treated LETO group, 40% (10/25 rats) in the BOP-treated OLETF group and 96% (24/25 rats) in the BOP-treated LETO group.

Figure 1 shows body weight curves during the study. The mean weight of OLETF rats treated with or without BOP increased until week 30 and was significantly heavier than that of LETO groups from weeks 5 to 30. The average food consumption (g/day per rat) of OLETF rats treated with BOP (32.6 ± 3.3, $P < 0.001$) or saline (32.9 ± 3.2, $P < 0.001$) was significantly greater than that of their LETO counterparts given BOP (20 ± 1.2) or saline (20.7 ± 1.1). BOP treatment did not affect final body weights and food consumption in either OLETF or LETO animals.

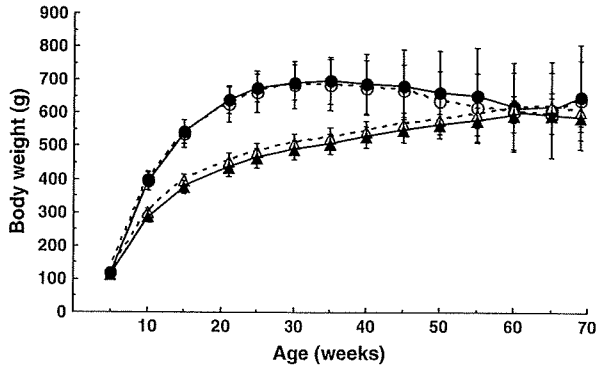

Fig. 1. Growth curves for OLETF (circles) and LETO (triangles) rats receiving BOP (closed symbols) or saline (open symbols).

Data on serum levels of phospholipid, triglyceride, total cholesterol, insulin and glucose are summarized in Table I. Those values for the OLETF rats were significantly higher than those of the LETO rats. The data suggested the OLETF rats to be hyperlipidemic and hyperglycemic, in a type II diabetic state, as reported previously (8,9). The serum glucose levels of the BOP-treated OLETF rats (208–608 mg/dl) were higher than those of the saline-treated OLETF rats (109–384 mg/dl), though the difference was not statistically significant ($P = 0.06$). Any influences of BOP treatment on the other values were not observed.

Carcinogenicity of BOP in OLETF and LETO rats

As summarized in Table II, neoplasms developed in a variety of tissues of the BOP-treated OLETF and LETO rats, including the thyroid, colon, liver, lungs, kidney, pancreas and subcutis. The highest yield was for thyroid tumors in the BOP-treated OLETF rats. The incidence was significantly higher than in the BOP-treated LETO group, being 96 and 71%, respectively. Pancreatic neoplasms developed in the BOP- and saline-treated OLETF rats at similar incidences. In each case, four pancreatic tumors were found, histopathologically diagnosed as one ductal adenocarcinoma and three acinar cell adenocarcinomas. In the LETO rats treated with BOP or saline, no pancreatic tumors were observed. In the tissues other than thyroid and pancreas, only few tumors developed in both strains (Table II). They included one colonic adenocarcinoma, one liver cell adenoma, one liver cell carcinoma, four lung adenomas and two renal fibrosarcomas in the BOP-treated OLETF rats, and two liver cell adenomas, eight lung adenomas and two renal cell adenomas, four renal cell adenocarcinomas, one renal mesenchymal tumor and one renal rhabdomyosarcoma of the BOP-treated LETO rats. However, there are no differences of the incidences in any of these neoplasms between the OLETF and LETO groups.

Enhanced development of BOP-induced thyroid cancer in OLETF rats

Thyroid neoplasms developing in the BOP-treated OLETF and LETO rats were histopathologically diagnosed as follicular cell adenomas (Figure 2A), follicular cell adenocarcinomas (Figure 2B and C) and anaplastic carcinomas (Figure 2D). In addition to spindle cell type of anaplastic carcinoma (Figure 2D), the coexistence of a type that consisted of spindle neoplastic cells and foci of follicular cell carcinoma with zones of transition was observed. As summarized in Table III, the incidences of BOP-induced thyroid follicular cell adenomas, poorly differentiated/anaplastic thyroid carcinoma, total carcinomas and total tumors (adenomas plus carcinomas) were significantly greater in the OLETF than the LETO rats. The incidence of well-/moderately differentiated thyroid carcinoma was also greater in the OLETF rats than the LETO rats, but the difference was not statistically significant. The multiplicities of follicular cell adenoma, well-/moderately differentiated follicular carcinomas, poorly differentiated/anaplastic follicular adenocarcinomas, total carcinomas and total tumors (adenomas plus carcinomas) in the thyroid were all significantly higher in the OLETF than the LETO rats. While the highest incidences of malignant epithelial neoplasms were for well-/moderately differentiated follicular cell carcinoma in both OLETF and LETO rats, large anaplastic carcinomas developed only in the OLETF rats and invaded into the surrounding tissues, including muscle, esophagus, trachea and lung.

The weights of thyroid glands were 1.5 times heavier in the saline-treated OLETF rats than in the saline-treated LETO rats, and BOP treatment significantly increased the weights for each strain (Table IV). The thyroid glands of BOP-treated OLETF rats were four times heavier than those of BOP-treated LETO rats. In addition, fibrosis accompanied by inflammation was prominent in the BOP-treated OLETF rats with thyroid carcinomas (Figure 2C) when compared with the BOP-treated LETO rats (Table IV).

Values for T3, T4 and TSH are shown in Table V. The T3 level in the OLETF rats was significantly higher than in their LETO counterparts, with or without BOP. The T4 level was increased by BOP

Table I. Serum lipid, insulin and glucose levels in OLETF and LETO rats given BOP or saline

Strain	Treatment	No. of rats	Triglyceride (mg/dl)	Total cholesterol (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)	Phopholipids (mg/dl)	FFA (mEq/l)	Insulin (ng/ml)	Glucose (mg/dl)
OLETF	BOP	23	433 ± 323[ab]	309 ± 124[ab]	71 ± 30[ab]	62 ± 36[ab]	399 ± 149[ab]	1041 ± 859[ac]	1.24 ± 1.28[ab]	391 ± 160[cd]
	Saline	22	535 ± 268[b]	331 ± 77[b]	70 ± 13[b]	63 ± 24[b]	430 ± 91[b]	1029 ± 432[c]	0.83 ± 0.94[c]	203 ± 106[e]
LETO	BOP	24	93 ± 30	135 ± 13	29 ± 3	23 ± 5	182 ± 25	533 ± 167	0.21 ± 0.20	130 ± 55
	Saline	24	95 ± 33	132 ± 20	29 ± 5	23 ± 4	171 ± 23	510 ± 132	0.26 ± 0.33	90 ± 9

HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; FFA, free fatty acids.
[a]Means ± SDs (n = 22–24).
[b]Significantly different from the LETO group with each treatment at $P < 0.001$.
[c]Significantly different from the LETO group with each treatment at $P < 0.01$.
[d]Means ± SDs (n = 5).
[e]Significantly different from the LETO group with each treatment at $P < 0.05$.

Table II. Tumor incidences in OLETF and LETO rats treated with BOP or saline

Strain	Treatment	Effective number of rats[a]	Number (%) of rats with tumors						
			Thyroid	Colon	Liver	Lung	Kidney	Pancreas	Subcutis
OLETF	BOP	24	23[b] (96)	1 (4)	2 (8)	4 (17)	2 (8)	4 (17)	0
	Saline	25	0	0	0	0	0	4 (16)	1 (4)
LETO	BOP	24	17 (71)	0	2 (8)	8 (33)	8 (33)	0	0
	Saline	25	0	0	0	0	0	0	0

[a]Number of animals that survived beyond week 44 (49 weeks of age).
[b]Significantly different from the LETO-BOP group at $P = 0.0240$.

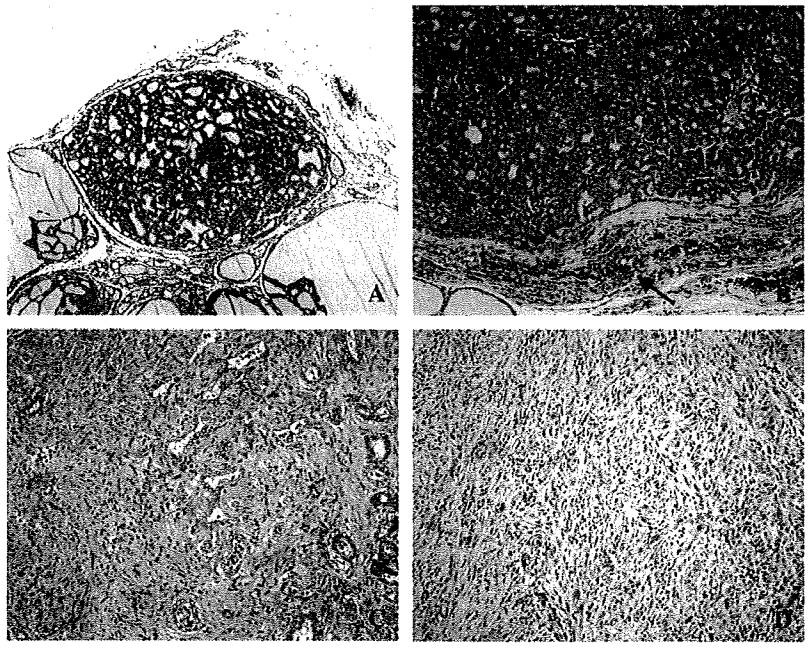

Fig. 2. Histopathology of thyroid proliferative lesions (**A–D**) developing in male OLETF rats receiving BOP. (**A**) A follicular cell adenoma. Tumor cells with slight nuclear atypia show a papillary to follicular cell pattern. The tumor is well demarcated by a fibrous capsule and compresses adjacent follicles. (**B**) A follicular cell carcinoma. The neoplastic cells with cellular pleomorphism form irregular follicles. Minimal invasion (arrow) is seen along one margin. (**C**) A follicular cell carcinoma with a scirrhous response. The tumor cells with nuclear atypia show irregular and follicular (tubular) growth patterns with extensive fibrosis. (**D**) An anaplastic carcinoma of spindle cell type. The tumor consists of pleomorphic spindle cells that show striform patterns, closely simulating the appearance of malignant fibrous histiocytoma. Hematoxylin and eosin stain. Original magnification: ×4 for A; ×10 for (**B–D**).

treatment in both strains, especially in the LETO rats and was consistently lower in OLETF than LETO rats. There were no differences in the TSH levels between the OLETF and LETO rats treated with or without BOP.

Colon carcinogenesis in OLETF rats

As mentioned, only one adenocarcinoma developed in the colon of a rat from the BOP-treated OLETF group. We further analyzed the number of ACF, putative precursor lesions (19), in order to determine

Table III. Histopathological diagnosis of thyroid tumors in the OLETF and LETO rats receiving BOP

Strain	Incidence[a] (%)					Multiplicity[b]				
	Follicular cell adenoma	Follicular cell adenocarcinoma		Total carcinoma	Total tumor	Follicular cell adenoma	Follicular cell adenocarcinoma		Total carcinoma	Total tumor
		Well- or moderately differentiated carcinoma	Poorly differentiated or anaplastic carcinoma				Well- or moderately differentiated carcinoma	Poorly differentiated or anaplastic carcinoma		
OLETF	19/24[b] (79)	20/24 (83)	10/24[c] (42)	22/24[d] (92)	23/24[e] (96)	1.38 ± 1.06[fg]	3.25 ± 2.21[g]	0.46 ± 0.59[h]	3.71 ± 2.26[g]	5.08 ± 2.86[g]
LETO	12/24 (50)	15/24 (63)	1/24 (4)	15/24 (63)	17/24 (71)	0.50 ± 0.51	0.96 ± 0.91	0.04 ± 0.20	1.00 ± 0.93	1.50 ± 1.25

[a]Number of rats with thyroid tumors/effective number of rats
[b]Significantly different from the LETO-BOP group at $P = 0.0344$.
[c]Significantly different from the LETO-BOP group at $P = 0.0022$.
[d]Significantly different from the LETO-BOP group at $P = 0.0182$.
[e]Significantly different from the LETO-BOP group at $P = 0.0240$.
[f]Means ± SDs.
[g]Significantly different from the LETO-BOP group at $P < 0.001$.
[h]Significantly different from the LETO-BOP group at $P < 0.005$.

Table IV. Mean weights of thyroid glands and scores for fibrosis in the OLETF and LETO rats

Strain	Treatment	Weight of thyroid glands (g)	Score of fibrosis
OLETF	BOP	1.026 ± 0.871[ab] ($n = 18$)	2.50 ± 1.32[b] ($n = 24$)
	Saline	0.045 ± 0.016[c] ($n = 22$)	0.05 ± 0.22[c] ($n = 25$)
LETO	BOP	0.251 ± 0.511[d] ($n = 24$)	1.25 ± 0.90[e] ($n = 24$)
	Saline	0.031 ± 0.005 ($n = 24$)	0.08 ± 0.28 ($n = 25$)

[a]Means ± SDs.
[b]Significantly different from the OLETF-saline, LETO-BOP and LETO-saline groups at $P < 0.001$ for each comparison.
[c]Significantly different from the LETO-BOP group at $P < 0.001$.
[d]Significantly different from the LETO-saline group at $P < 0.001$
[e]Significantly different from the LETO-saline group at $P < 0.05$.

Table V. Serum thyroid hormone and TSH levels in OLETF and LETO rats

Strain	Treatment	No. of rats	T3 (ng/ml)	T4 (μg/dl)	TSH (ng/ml)
OLETF	BOP	23	1.2 ± 0.3[abc]	3.3 ± 0.5[b]	5.9 ± 1.7
	Saline	22	1.1 ± 0.2[de]	3.0 ± 0.7[b]	7.0 ± 2.1
LETO	BOP	24	0.9 ± 0.1	4.2 ± 0.5[c]	5.9 ± 3.4
	Saline	24	0.9 ± 0.1	3.3 ± 0.4	6.0 ± 2.6

[a]Means ± SDs.
[b]Significantly different from the LETO-BOP group at $P < 0.001$.
[c]Significantly different from the LETO-saline group at $P < 0.001$.
[d]Significantly different from the LETO-BOP group at $P < 0.01$.
[e]Significantly different from the LETO-saline group at $P < 0.01$.

the colon sensitivity to BOP in the OLETF and LETO rats. BOP treatment induced colonic ACF in both strains and a significantly higher incidence was evident in the OLETF rats (100 versus 63% in the LETO rats, Table VI). The multiplicity of colonic ACF in the BOP-treated OLETF group was also significantly higher than in the BOP-treated LETO group.

Discussion

In the present experiment, BOP induced tumors in a variety of organs, such as thyroid, colon, liver, lungs and kidneys of OLETF and LETO rats, but did not significantly increase pancreatic ductal carcinoma development. The incidence and multiplicity of thyroid cancer in the

Table VI. Incidences and multiplicity of colonic lesions in the OLETF and LETO rats treated with BOP

Strain	No. of rats	Incidence (%)		Multiplicity	
		ACF	Carcinoma	ACF	Carcinoma
OLETF	17	17 (100)[a]	1 (6)	14 ± 11[bc]	0.06 ± 0.24
LETO	19	12 (63)	0	1 ± 1[b]	0

[a]Significantly different from the LETO-BOP group at $P = 0.0084$.
[b]Means ± SDs.
[c]Significantly different from the LETO-BOP group at $P < 0.001$.

BOP-treated OLETF group were the highest among the tumors developing and significantly greater than in the BOP-treated LETO rats. Of particular interest, anaplastic thyroid carcinomas were developed in BOP-treated OLETF rats with hyperglycemia and hyperlipidemia, but not in any of the LETO rats. Our results thus suggest that diabetes mellitus and/or hyperlipidemic status affect carcinogenicity and promote cancer development in the thyroid. Since a history of diabetes mellitus is reported to be a risk factor for anaplastic thyroid cancer in humans (3), it is probable that the biochemical status in the body accelerates transformation from differentiated to anaplastic tumors.

Thyroid cancer is a relatively rare cancer, representing ~1% of all malignancies and accounting for ~0.5% of all deaths caused by malignant tumors (20). The well-differentiated thyroid carcinoma (21) is generally characterized by slow growth and a low mortality rate (8–15%) (22–26) and is only infrequently a cause of death. In contrast, anaplastic thyroid carcinomas, 5–15% of all thyroid cancers, are responsible for most thyroid cancer deaths (27,28), with only short survival periods (29,30). Such a histologic type of thyroid cancer usually occurs in elderly patients as a rapidly growing mass associated with dyspnea, dysphagia and hoarseness. In most of the cases, extrathyroidal extension is observed at the time of initial presentation; growth of anaplastic thyroid carcinoma is very rapid with infiltration of surrounding muscle, esophagus, trachea, skin and even bone. The fact that poorly differentiated/anaplastic thyroid carcinomas more frequently developed in the BOP-treated OLETF rats is therefore of major interest. The pathogenesis of anaplastic thyroid carcinoma is not completely understood—it could arise *de novo* or from a preexisting well-differentiated thyroid carcinoma. Most aggressive tumors, however, arise as a result of anaplastic transformation of a preexisting well-differentiated tumor, papillary or follicular carcinoma, as found in this study, although they histologically mimic sarcoma or carcinosarcoma (31). Immunohistochemistry of anaplastic thyroid carcinomas found in this study showed negative to weak positivity against an

antibody of thyroid transcription factor-1 in tumor nuclei (data not shown), as in human cases (32). In the present study, fibrosis was also remarkable in follicular adenocarcinomas of the BOP-treated OLETF rats, suggesting the presence of factors promoting stromal cell proliferation.

As indicated in an *N*-bis(2-hydroxypropyl)nitrosamine-induced thyroid cancer model using F344 rats, where decreased T3 and T4 levels and an increased TSH level in the serum were described (33), thyroid hormones participate in thyroid carcinogenesis. Studies on tumor-promoting effects of *p*-aminobenzoic acid, xylazine and β-estradiol 3-benzoate on thyroid follicular cells suggest an involvement of inhibition of thyroid iodine uptake and organification, resulting in serum TSH stimulation dependent on continuous reduction of serum T4 levels through the feedback system in the pituitary–thyroid axis (34–36). In the present study, the incidence of thyroid carcinoma in the BOP-treated OLETF group that exhibited mild hypothyroidism with hyperlipidemia was significantly higher than in the BOP-treated LETO group. These results suggest that disrupted thyroid hormone levels and diabetes mellitus/hyperlipidemic status in concert affect carcinogenicity and promote thyroid cancer development in the OLETF rats. In fact, clinically, association between Hashimoto thyroiditis and thyroid cancer (37), and that between subclinical hypothyroidism and hyperlipidemia (38) is reported.

Epidemiological data suggest a clear association between colon cancer development and hypertriglycemia/hypercholesteronemia (4,5) and our previous study revealed that APC^{1309} and Min mice, known to feature a hyperlipidemic state, develops more numerous polyps in their intestinal tracts than do wild-type mice (39,40). In the current study, the colons of OLETF rats that were hypertriglycemic and hyperlipidemic appeared more sensitive to carcinogen than those of LETO rats based on the results for ACF that are putative precursor lesions for colon adenocarcinomas (19). The findings are consistent with a recent report that type II diabetes mellitus may enhance the generation and growth of colon carcinoma in OLETF rats treated with 1,2-dimethylhydrazine (41).

In conclusion, our findings described here indicate that a diabetic and/or hyperlipidemic state could enhance BOP-induced carcinogenesis of the thyroid gland and colon, in particular the thyroid, in OLETF rats. This strain can thus be considered as good model animals for investigating the relation between diabetes/hyperlipidemia and development of cancer.

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Plasminogen activator inhibitor-1 (Pai-1) blockers suppress intestinal polyp formation in Min mice

Michihiro Mutoh*, Naoko Niho, Masami Komiya, Mami Takahashi, Rina Ohtsubo, Kiyoshi Nakatogawa[1], Kentaro Ueda[1], Takashi Sugimura and Keiji Wakabayashi

Cancer Prevention Basic Research Project, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan and [1]Shizuoka Coffein Co. Ltd, 129 Suidocho, Shizuoka-shi 420-0008, Japan

*To whom correspondence should be addressed. Tel: +81 3 3542 2511; Fax: +81 3 3543 9305;
Email: mimutoh@gan2.ncc.go.jp

Obesity and hyperlipidemia are known to increase colorectal tumor risk. We noticed that Min mice, featuring a defect in the *adenomatous polyposis coli* (*Apc*) gene, develop intestinal polyps along with high serum triglyceride (TG) levels up to 10-fold those observed in wild-type mice. In these mice, messenger RNA (mRNA) expression of lipoprotein lipase, which catalyzes hydrolysis of TG, is downregulated. In the present study, we focused on adipocytokines, especially plasminogen activator inhibitor-1 (Pai-1), which is involved in hyperlipidemic status and may promote intestinal polyp formation in Min mice. Serum Pai-1 levels in the 15-week-old male Min mice were eight times higher than in wild-type mice and hepatic Pai-1 mRNA levels were 11-fold increased. In addition, Pai-1 immunostaining was strong in small intestinal epithelial cells of Min mice. Administration of a PAI-1 inhibitor, SK-216, at 25, 50 and 100 p.p.m. doses in the diet for 9 weeks reduced serum Pai-1 levels and hepatic Pai-1 mRNA levels of Min mice to the wild-type levels. Moreover, SK-216 at 50 and 100 p.p.m. significantly reduced total numbers of intestinal polyps to 64 and 56% of the untreated group value, respectively. Serum TG levels were also decreased by 43% at the dose of 100 p.p.m. Administration of 50 p.p.m. SK-116, another PAI-1 inhibitor, for 9 weeks similarly reduced serum Pai-1 levels and total numbers of intestinal polyps to 70% of the untreated group value. These results indicate that Pai-1 induction associated with hypertriglyceridemia may contribute to intestinal polyp formation with *Apc* deficiency, and PAI-1 could thus be a novel target for colorectal chemopreventive agents.

Introduction

Colon cancer is one of the most common solid cancers, has an increasing incidence in developed countries and is now widely considered to be obesity associated (1,2). Hyperlipidemia, especially hypertriglyceridemia, is known to increase the risk of colorectal tumor (3–5) and recently we reported an age-dependent hyperlipidemic state in *adenomatous polyposis coli* (*Apc*)-deficient Min and *Apc*1309 mice, animal models of familial adenomatous polyposis (6–8). Familial adenomatous polyposis is characterized by the appearance of hundreds of adenomatous polyps in the colon and rectum due to germ line mutations of the *APC* gene. Truncating mutations in *APC* activate Wnt signaling to promote cell growth and are observed in almost 70% of the sporadic colorectal cancers (9).

Although the direct link between *APC* deficiency and hyperlipidemia has yet to be clarified, it is notable that serum triglyceride (TG) levels in *Apc*-deficient mice are almost 10-fold than those in wild-type littermates, this appearing due to low messenger RNA (mRNA) expression levels for lipoprotein lipase (LPL), which catalyzes hydrolysis of TG (6). We have demonstrated that induction of LPL mRNA by peroxisome proliferator-activated receptor-α and -γ agonists and a selective LPL-inducing agent, NO-1886, which lacks potential for activating the peroxisome proliferator-activated receptor pathways, suppressed both the hyperlipidemic status and intestinal polyp formation (6–8). Adipocytokines upregulated in the metabolic syndrome, also associated with a hyperlipidemic status, may be factors impacting on intestinal polyp formation. During investigation of expression levels of adipocytokines in Min mice, we have occasionally detected remarkable elevation of hepatic plasminogen activator inhibitor-1 (Pai-1) expression.

PAI-1 is a direct binding primary inhibitor of plasminogen activators, uPA and tPA, and is known to be induced by TG, very low-density lipoprotein (TG-rich lipoprotein), transforming growth factor β, various growth factors, tumor suppressor p53 and Nuclear factor kappa B (NFκB) (10–12). Moreover, a very low-density lipoprotein response element has been identified in the promoter region of the PAI-1 gene locus that mediates very low-density lipoprotein-induced PAI-1 transcription in endothelial cells (13). PAI-1 can also inhibit activation of metalloproteinases via inhibition of plasmin production from plasminogen. Metalloproteinases degrade extracellular matrix proteins, which modulate cellular adhesion and migration (14,15). In contrast to its antiproteolytic activity, it has been reported that PAI-1 promotes cancer invasion and metastasis (16). Furthermore, PAI-1 could modulate cell proliferation and stimulate angiogenesis. In one experiment using Pai-1-deficient mice, Pai-1 deficiency abolished cancer invasion and angiogenic activity (17). In this context, it should be mentioned that PAI-1 is significantly upregulated in neoplastic tissue of the human colon (18). The PAI-1 promoter 4G/5G polymorphism, in which the 4G allele is associated with high PAI-1 expression, may influence the development of aggressive fibromatosis in familial adenomatous polyposis patients (19). Thus, it is conceivable that Pai-1 is one of the factors that explain linkages between hyperlipidemia and intestinal tumorigenesis.

In this study, we examined the effects of Pai-1 inhibitors, SK-216 and SK-116, in the diet on both hyperlipidemia and intestinal polyp formation in Min mice and demonstrated concomitant suppression of both. The possible mechanisms of its action in *Apc*-deficient mice and usage of PAI-1 inhibitors as possible candidates for colon cancer prevention are also discussed.

Materials and methods

Cell culture

Azoxymethane-induced F344 rat colon cancer-derived cell line RCN-9, which was obtained from RIKEN Bioresource Center (Tsukuba, Japan), was used. RCN-9 cells were grown in Dulbecco's modified Eagle medium supplemented with 5% fetal bovine serum at 37°C in a humidified incubator with 5% CO_2.

Animals and chemicals

Male C57BL/6-*ApcMin*/+ mice (Min mice) were purchased from The Jackson Laboratory Maine, USA at 6 weeks of age and genotyped as previously reported (20). Heterozygotes of the Min strain and wild-type (C57BL/6J) mice were 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 h light/dark cycle. The PAI-1 inhibitors SK-216, disodium [5-[[6-[5-(1,1-dimethylethyl-2-benzoxazolyl)-2-naphthalenyl]oxy] pentyl] propanedioate, and SK-116, disodium [5-[[6-[4-phenyl-6-(phenylmethoxy)-2-pyrimidinyl]-2-naphthalenyl]oxy] pentyl]propanedioate, were chemically synthesized at Shizuoka Coffein Co. Ltd. Their structures are shown in Figure 1. *In vitro* data showed that these compounds reverse the inhibitory effects of PAI-1 against both tPA and urokinase using the method described by Charlton *et al.* (21). IC_{50} for SK-216 is 44 μM and that for SK-116 is 35 μM were reported in international patent WO04/

Abbreviations: Ab, antibody; APC, adenomatous polyposis coli; LPL, lipoprotein lipase; mRNA, messenger RNA; Pai-1, plasminogen activator inhibitor-1; PCR, polymerase chain reaction; RT, reverse transcription; TG, triglyceride.

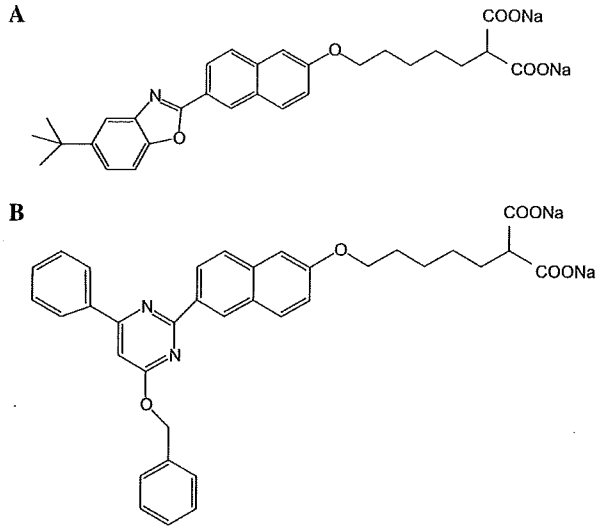

Fig. 1. Structures of SK-216 (**A**) and SK-116 (**B**).

010996 and WO04/011442, respectively. SK-216 was well mixed at concentrations of 25–100 p.p.m. in AIN-76A powdered basal diet (CLEA Japan, Tokyo, Japan). SK-116 was also well mixed at 50 p.p.m. in the same way.

Animal experiments

To investigate the effects of PAI-1 inhibitors on intestinal polyp formation, 10 male Min mice at 7 weeks of age were given SK-216 and SK-116 in the diet for 8 weeks. Food and water were available *ad libitum*. The animals were observed daily for clinical signs and mortality. Body weights and food consumption were measured weekly. Animals were anesthetized with ether and killed, and blood samples were collected from the abdominal aorta. Serum levels of TG and total cholesterol were measured as reported (6). The experiments were conducted according to the Guidelines for Animal Experiments in National Cancer Center of the Committee for Ethics of Animal Experimentation of the National Cancer Center.

The intestinal tract was removed, filled with 10% buffered formalin and separated into the small intestine, cecum and colon. The small intestine was divided into the proximal segment (4 cm in length) and then the proximal (middle) and distal halves of the remainder. All segments were opened longitudinally and fixed flat between sheets of filter paper in 10% buffered formalin. The numbers and sizes of polyps and their distributions in the intestine were assessed with a stereoscopic microscope (6).

To investigate the effects of PAI-1 inhibitors on Pai-1 expression levels in mouse intestinal mucosa, C57/BL6 mice were purchased from CLEA Japan at 6 weeks of age and were given either 0 or 100 p.p.m. SK-216 for 1 week. A day before killing, 200 μl soy oil (Wako Pure Chemical Industries, Ltd, Osaka, Japan) was given by gavage to the mice. The intestinal mucosa was removed by scraping for further reverse transcription (RT)–polymerase chain reaction (PCR) analysis.

Real-time PCR analysis and RT–PCR analysis

Tissue samples from liver of mice were rapidly deep-frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated from tissues by using Isogen (Nippon Gene, Tokyo, Japan), treated with DNase (Invitrogen) and 3 μg aliquots in a final volume of 20 μl were used for synthesis of cDNA using an Omniscript RT Kit (Qiagen, Hilden, Germany) and an oligo(dT) primer. Real-time PCR was carried out using a DNA Engine Option ™ 2 (MJ Japan Ltd, Tokyo, Japan) with SYBR Green Realtime PCR Master Mix (Toyobo Co., Osaka, Japan) according to the manufacturer's instructions. Primers for mouse Pai-1 (5′primer-GACACCCTCAGCATGTTCATC and 3′primer-AGGGTTGCACTAAACATGTCAG) and glyceraldehyde-3-phosphate dehydrogenase (5′primer-TTGTCTCCTGCGACTTCA and 3′primer-CACCACCCTGTTGCTGTA) were employed (22). To assess the specificity of each primer set, amplicons generated from the PCR reaction were analyzed for melting curves and also by electrophoresis in 2% agarose gels.

Tissue samples from intestinal mucosa were treated the same as liver samples and 1 μl of cDNA was included in a final volume of 10 μl with a PTC-200 DNA Engine (MJ Research, Waltham, MA) by using a Omniscript RT Kit (Qiagen). Cycling conditions were as follows: 94°C for 5 s, annealing temperature (60°C) for 30 s, 72°C for 60 s and 32 cycles after an initial step of 95°C for 3 min. A final elongation step of 72°C for 10 min completed the PCR. The products were then electrophoresed on 2% agarose gels.

Immunohistochemical staining

Small intestines were fixed, embedded and sectioned as Swiss rolls for further immunohistochemical examination with the avidin–biotin complex immunoperoxidase technique and polyclonal rabbit anti-Pai-1 antibodies (Abs) (Santa Cruz Biotechnology, Santa Cruz, CA) at 100× dilution. As the secondary Ab, biotinylated horse anti-rabbit IgG, affinity purified was employed at 200× dilution. Staining was performed using avidin–biotin reagents (Vectastain ABC reagents; Vector Laboratories), 3,3′-diaminobenzidine and hydrogen peroxide, and the sections were counterstained with hematoxylin to facilitate orientation. As a negative control, consecutive sections were immunostained without exposure to the primary Ab.

Enzyme-linked immunosorbent assay

The concentration of Pai-1 in the plasma was determined using a MOUSE PAI-1 Total Antigen ELISA Kit (Innovative Research, MI, USA) for five samples each from wild-type mice, untreated Min and PAI-1 inhibitor-treated Min mice, according to the manufacturer's protocol.

Western blot analysis

Protein expression was analyzed by western blot. Cells (2×10^5) were seeded in 24-well plates. After treatment, cells were lysed in 100 μl lysis buffer [0.0625 M Tris–HCl (pH 6.8), 20% 2-mercaptoethanol, 10% glycerol, 5% sodium dodecyl sulfate]. Equal amounts of protein were separated in 10% polyacrylamide gel electrophoresis–sodium dodecyl sulfate gels and transferred onto polyvinylidene difluoride membranes (Millipore, MA). Abs against the Pai-1 (Santa Cruz Biotechnology) were used at a 1:2000 dilution. Peroxidase-conjugated secondary Abs for anti-rabbit IgG were obtained from GE Healthcare, Buckingham shire, UK. Blots were developed with enhanced chemiluminescence western blotting detection reagents (Amersham Biosciences, Buckingham shire, UK).

NFκB–DNA-binding activity assay

The activity of NFκB binding to oligonucleotides containing an NFκB consensus binding sites was measured by using TransAM™ NFκB Transcription Assay Kits according to the manufacturer's instructions (ActiveMotif, CA, USA). Briefly, RCN-9 cells treated with 50 μM SK-216 for 6 h were harvested from 24-well plates, and nuclear fractions were isolated by using a Nuclear Extract Kit from ActiveMotif. Nuclear fractions were applied to 96-well plates coated with oligonucleotides containing an NFκB consensus binding site. After treatment with anti-NFκB Ab, another peroxidase-linked Ab specific for NFκB was added. The substrate solution was added and optical density was assessed using the microplate reader set to 450 nm.

Statistical analysis

All the results are expressed as mean ± standard error values, with statistical analysis using Dunnett's test, except for the serum Pai-1 level investigation in Figure 2A. The Mann–Whitney test was used for statistical analyses of the serum Pai-1 level. Differences were considered to be statistically significant at $P < 0.05$.

Results

Expression of Pai-1 in serum, liver and small intestine

Serum Pai-1 was obviously increased (approximately eight times) in Min mice at 15 weeks of age as compared with wild-type littermates (Figure 2A). Pai-1 mRNA levels in the Min mice livers were also almost 11-fold elevated (Figure 2B). Furthermore, expression of Pai-1 was detected in small intestinal epithelial cells, but appeared weaker in stromal cells of Min mice (Figure 2C). Expression of Pai-1 was similar in both polyp and non-tumorous epithelial cells in the intestine (Figure 2D). Pai-1 immunostaining was strong in small intestinal epithelial cells of Min mice, but slightly weak in wild-type littermates, and no artificial stain was detected in controls using the second Ab only (data not shown).

Suppression of intestinal polyp formation in Min mice by Pai-1 inhibitors

Administration of SK-216 at 25, 50 and 100 p.p.m. for 9 weeks did not affect body weights, food intake or clinical signs of Min mice

throughout the experimental period. Average daily food intake did not differ significantly among the groups, being 3.1, 3.4, 3.1 and 3.0 g per mouse per day for the 0, 25, 50 and 100 p.p.m. groups of Min mice, respectively. During the experiment, severe hemorrhage, which may occur with fibrinolysis, was not observed in both Pai-1 inhibitor-treated or -untreated mice. In addition, there were no changes observed in any organ weights that might have been attributable to toxicity.

Table I summarizes data for the number and distribution of intestinal polyps in the basal diet and SK-216 treated groups. Almost all polyps developed in the small intestine, with only a few in the colon (6). The total number of polyps was significantly decreased by administration of 50 and 100 p.p.m. SK-216 to 64 and 56% of the untreated control value, respectively. Reduction in the proximal, middle and distal parts was by 56, 48 and 28% with 50 p.p.m. and by 63, 59 and 28% with 100 p.p.m., respectively. Treatment with SK-216 did not affect the numbers of colon polyps. Administration of 50 p.p.m. SK-116 also decreased the total number of polyps to 70% of the untreated control value with significant reduction in the proximal section (Table II).

Figure 3 shows the size distributions of intestinal polyps in the basal diet, SK-216-and SK-116-treated groups. The maximal number of polyps was observed in the size range between 0.5 and 3.0 mm in diameter. Administration of 50 and 100 p.p.m. SK-216 reduced the numbers of polyps of all sizes (Figure 3A). Administration of 50 p.p.m. SK-116 reduced the numbers of polyps sized <1.5 mm in diameter (Figure 3B).

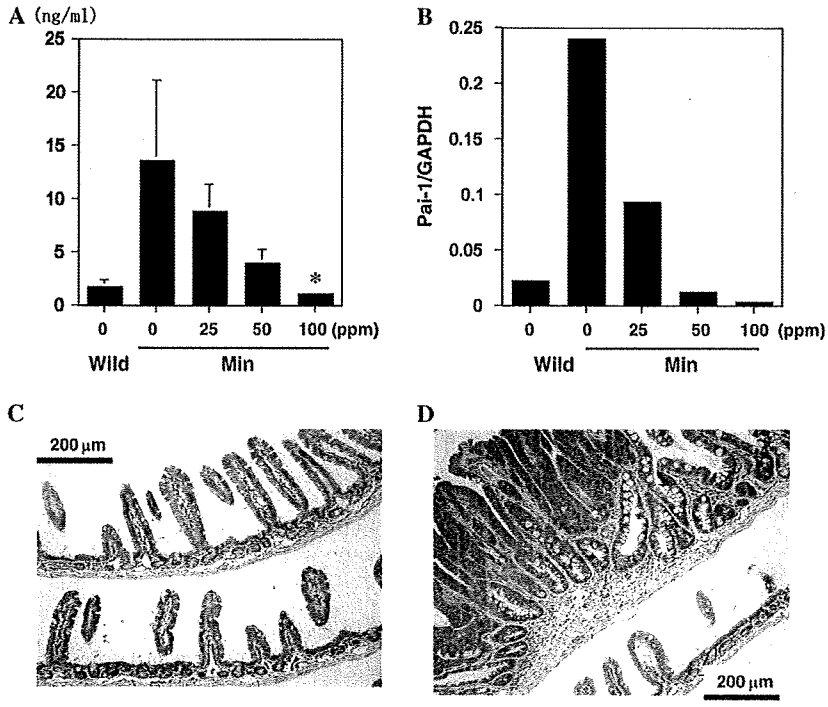

Fig. 2. Changes of serum Pai-1 level, liver Pai-1 mRNA and protein expression in the small intestine of Min mice. (**A**) Serum Pai-1 levels were detected by enzyme-linked immunosorbent assay using samples from Min mice ($n = 5$ each group) given diets containing SK-216 at doses of 25–100 p.p.m. for 9 weeks and also that from untreated wild-type mice ($n = 5$). Data are means ± standard errors $^*P < 0.05$. (**B**) Quantitative real-time PCR analysis of Pai-1 mRNA expression in the livers of Min mice given diets containing SK-216 at doses from 25–100 p.p.m. for 9 weeks and also that in untreated wild-type mice. Data are mean of two mice of each group. (**C**) Immunohistochemistry of Pai-1 expression in normal parts of the small intestine of a Min mouse. (**D**) Immunohistochemistry of Pai-1 expression in a polyp part of the small intestine in a Min mouse. Bars represent 200 μm. Data are representative of six mice.

Table I. Suppression of intestinal polyp development in Min mice by SK-216

No. of polyps/mouse		Small intestine			Colon	Total
Group (p.p.m.)	No. of mice	Proximal	Middle	Distal		
0	10	5.2 ± 0.7[a]	19.7 ± 2.4	40.6 ± 4.1	0.5 ± 0.3	67.0 ± 4.8
25	10	3.6 ± 0.6 (69)[b]	15.6 ± 2.4 (79)	44.5 ± 5.8 (110)	1.6 ± 0.3	65.3 ± 7.6 (97)
50	10	2.3 ± 0.4[c] (44)	10.3 ± 1.2[c] (52)	29.1 ± 2.9 (72)	1.3 ± 0.3	43.1 ± 4.0[c] (64)
100	10	1.9 ± 0.4[c] (37)	8.1 ± 1.0[c] (41)	25.3 ± 1.6[d] (62)	0.7 ± 0.2	37.7 ± 2.5[c] (56)

[a]Data are means ± standard errors.
[b]Numbers in parentheses are percentages of the control basal diet values.
[c]Significantly different from the basal diet group at $P < 0.01$.
[d]Significantly different from the basal diet group at $P < 0.05$.

Reduction of serum Pai-1 levels and liver Pai-1 mRNA levels in Min mice by Pai-1 inhibitors

It has been recognized that Pai-1 inhibitors, SK-216 and SK-116, inhibit Pai-1 activity. The present study revealed that Pai-1 inhibitors suppress Pai-1 at both protein and mRNA levels. The highest dose used in this study, 100 p.p.m. SK-216, suppressed serum Pai-1 levels to the wild-type level (Figure 2A). Real-time PCR revealed that administration of 25, 50 and 100 p.p.m. SK-216 for 9 weeks suppressed increased hepatic Pai-1 mRNA levels (Figure 2B) in Min mice in a dose-dependent manner. Another Pai-1 inhibitor, SK-116, also reduced serum Pai-1 level in Min mice from 20.1 ± 6.7 ng/ml (0 p.p.m.) to 6.9 ± 4.1 ng/ml (50 p.p.m.). In the immunohistochemistry study, Pai-1 could be detected more weakly in non-tumorous and polyp epithelial cells of the small intestine in Min mice treated with 100 p.p.m. SK-216 compared with that of untreated Min mice (data not shown).

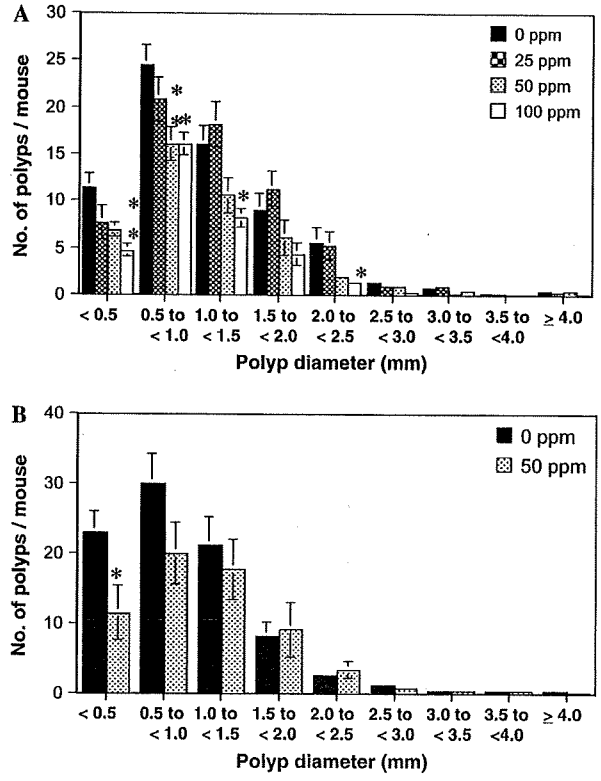

Fig. 3. Effects of SK-216 and SK-116 on the size distribution of intestinal polyps in Min mice. (**A**) Min mice were fed a basal diet (black filled box) or a diet containing 25 p.p.m. (large dotted box), 50 p.p.m. (dotted box) or 100 p.p.m. (open box) SK-216 for 9 weeks. (**B**) Min mice were fed a basal diet (black filled box) or a diet containing 50 p.p.m. (dotted box) SK-116 for 9 weeks. The number of polyps per mouse in each size class is given as a mean ± standard error. $^{*}P < 0.05$; $^{**}P < 0.01$.

Improvement of serum lipid levels in Min mice by Pai-1 inhibitors

Consistent with our previous reports (6–8), serum TG levels in the Min mice fed the basal diet at 15 weeks of age were higher at 117 mg/dl than the 39.2 mg/dl in wild-type mice (Figure 4). Total cholesterol levels in Min mice were also increased 1.3-fold (92 versus 62 mg/dl) while free fatty acid levels were almost the same in both Min and wild-type mice. Administration of 50 and 100 p.p.m. SK-216 decreased serum levels of TG in Min mice to 74 and 57% of the untreated control value, respectively (Figure 4). Administration of 50 and 100 p.p.m. SK-216 also decreased serum levels of TG in wild-type mice from 39.2 to 18.4 and 17.6 mg/dl ($P < 0.01$), respectively. The levels of total cholesterol and free fatty acid were not decreased by SK-216 treatment.

Reduction in serum TG levels was also observed in the 50 p.p.m. SK-116-treated group. Administration of 50 p.p.m. SK-116 decreased serum levels of TG in Min mice to 76% of the untreated control value.

Decrease of Pai-1 mRNA levels and NFκB binding activity in intestinal mucosa cells by Pai-1 inhibitor

To investigate whether the Pai-1 inhibitors directly targeted the intestinal mucosa, SK-216 in diet was administered to C57/BL6 mice. As shown in Figure 5A, treatment with soy oil, consisting of TG as a major component, increased Pai-1 expression levels in the intestinal mucosa of two out of three mice. A weeklong treatment with 100 p.p.m. SK-216 reduced Pai-1 mRNA levels to lower than non-treated mice in two out of three mice. Similar results were obtained in an *in vitro* study. Treatment with 50 μM SK-216 for 17 h reduced basal Pai-1 protein levels in the colon cancer cell line RCN-9 (Figure 5B). Moreover, 50 μM SK-216 treatment for 6 h decreased NFκB activity (Figure 5C). These results suggest that decreased NFκB activity may be involved in both suppression of Pai-1 mRNA and inhibition of polyp formation in Min mice by SK-216 treatment.

Discussion

This study provided evidence that administration of the PAI-1 inhibitors SK-216 and SK116, which also reduce Pai-1 mRNA and protein levels, suppresses intestinal polyp formation in Min mice. It is therefore speculated that Pai-1 activity itself may play an important role in intestinal polyp formation in *Apc*-deficient mice.

We previously reported markedly increased serum levels of TGs and low levels of LPL mRNA in liver and small intestine in Min mice compared with their wild-type counterparts (6–8). Thus, we hypothesized that hypertriglyceridemia is a leading cause of intestinal polyp

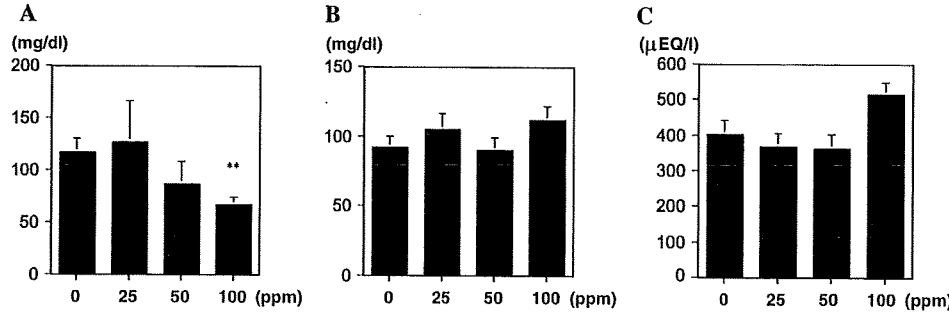

Fig. 4. Suppression of serum lipid levels in Min mice by SK-216. Values for serum levels of TG (**A**), total cholesterol (**B**) and free fatty acids (**C**) in Min mice given diet containing SK-216 at doses of 25–100 p.p.m. for 9 weeks are shown. Data are means ± standard errors. $^{**}P < 0.01$.

formation. However, the molecular mechanisms could only be partially addressed since only little information is available as to effects of TG-rich lipoproteins (23,24). TG-rich lipoproteins from type IV hyperlipidemic patients induce phosphorylation of p38 mitogen-activated protein kinase, and CAMP response element binding protein Inhibitor of kappa B α and activate DNA-binding activity of transcriptional factors, CREB, NFκB and AP-1. TG-rich lipoproteins also upregulate the expression of proinflammatory and adhesion-related genes, monocyte chemoattractant protein-1, interleukin-6, intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and PAI-1. These mitogen-activated protein kinase pathways and molecules are well known to be involved in endothelial cell growth. Treatment of smooth muscle cells with low-density lipoprotein results in the activation of protein kinase C and mitogen-activated protein kinase as well as induction of the cell cycle-related genes c-*fos*, c-*myc* (24) and early growth response gene-1 (*egr*-1, 25). Thus, hypertriglyceridemia may also modify epithelial cell growth. To explore molecular mechanisms underlying the link between hypertriglyceridemia and polyp formation, we first selected candidate molecules from those which are increased with the metabolic syndrome (26). Focusing on adipocytokines, we selected Pai-1 among possible candidate molecules, including adiponectin, IL-1, IL-6, leptin and tumor necrosis factor α. Liver was used for the RT–PCR analysis because this is the major Pai-1-producing organ and expression may correlate with hyperlipidemic states in the mice. Moreover, Pai-1 immunostaining was strong in small intestinal epithelial cells of Min mice. The reason why PAI-1 inhibitors also reduced serum TG levels remains unclear and examination of Pai-1 effects on TG metabolism would appear warranted.

Regarding the mechanisms underlying suppression of intestinal polyp formation by PAI-1 inhibitor in Min mice, contrary reports should be noted (10,27). Inhibition of PAI-1 activation results in generation of active growth factors from inactivated forms like heparin-bound epidermal growth factor, hepatocyte growth factor, basic fibroblast growth factor or insulin-like growth factors (10). Moreover, suppression of PAI-1 enhances growth factor signaling through the phosphatidylinositol 3-kinase–protein kinase B route (27). These reports indicate that PAI-1 may inhibit cell proliferation. However, Li *et al.* (28) have reported that genetic Pai-1 deficiency reduced the number of aggressive fibromatosis tumors in Apc/Apc1638N mice. The data from Li *et al.* were partially consistent with our results. Especially in male Pai-1-null Apc/Apc1638N mice, the number of aggressive fibromatosis tumors was decreased. However, no significant difference was observed in the number of gastrointestinal tumors compared with that of Apc/Apc1638N mice (0.7 ± 0.5 versus 1.1 ± 0.4; $P > 0.05$). These results may be due to weak statistical power derived from the relatively few intestinal polyps that developed in Apc/Apc1638N mice. Pai-$1^{-/-}$ tumor cells demonstrated reduced proliferation and motility *in vitro* (28). In addition, our *in vitro* study demonstrated that Pai-1 inhibitor SK-216 also decreased NFκB activity. This decrease in NFκB activity may be involved in both suppression of Pai-1 levels and inhibition of polyp development in Min mice.

In conclusion, this study indicated that the PAI-1 inhibitors, SK-216 and SK-116, have potential benefit for suppression of intestinal polyp development. Thus, SK-216, SK-116 and related derivatives could be promising candidate chemopreventive agents for colon cancer. As it is becoming increasingly clear that hyperlipidemia is an important player in carcinogenesis, our observations may lead to a better understanding of the role of hyperlipidemia in colon carcinogenesis.

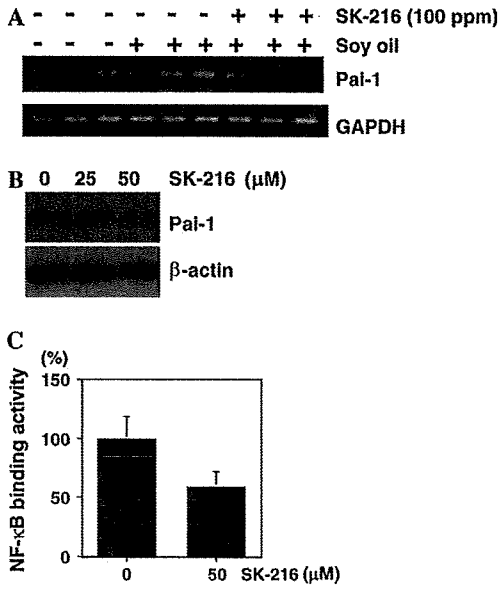

Fig. 5. Decrease of Pai-1 mRNA levels and NFκB-binding activity in intestinal mucosa/RCN-9 cells by SK-216 treatment. (**A**) RT–PCR for Pai-1 and GAPDH are shown. C57/BL mice ($n = 3$ for each group) were fed diet with or without 100 p.p.m. SK-216 for a week and gavaged with 200 μl soy oil 2 h before collecting intestinal mucosa. RT–PCR was performed as describe in Materials and Methods. (**B**) Western blotting for Pai-1 and beta-actin are shown. RCN-9 cells grown in 24-well plates were treated for 17 h with indicated doses of SK-216. Actin was used as a loading control. (**C**) RCN-9 cells grown in six-well plates were treated with and without 50 μM SK-216 for 6 h. Nuclear fractions of RCN-9 cells were isolated and analyzed for NFκB-binding activity as describe in Materials and Methods. Values represent means ± standard errors of three wells.

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Conflict of Interest Statement: None declared.

Table II. Suppression of intestinal polyp development in Min mice by SK-116

No. of polyps/mouse		Small intestine			Colon	Total
Group (p.p.m.)	No. of mice	Proximal	Middle	Distal		
0	10	4.8 ± 0.6[a]	23.4 ± 3.1	58.7 ± 7.2	1.9 ± 0.5	88.8 ± 10.3
50	10	2.0 ± 0.4[b] (42)[c]	16.2 ± 2.9 (69)	42.9 ± 7.2 (73)	1.0 ± 0.3	62.1 ± 9.5 (70)

[a]Data are means ± standard errors.
[b]Significantly different from the basal diet group at $P < 0.01$.
[c]Numbers in parentheses are percentages of the control basal diet values.

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Increased expression of inducible nitric oxide synthase (iNOS) in *N*-nitrosobis(2-oxopropyl)amine-induced hamster pancreatic carcinogenesis and prevention of cancer development by ONO-1714, an iNOS inhibitor

Mami Takahashi[1,*], Tsukasa Kitahashi[1], Rikako Ishigamori[1], Michihiro Mutoh[1], Masami Komiya[1], Hidetaka Sato[2], Yoshihisa Kamanaka[3], Masao Naka[3], Takayuki Maruyama[3], Takashi Sugimura[1] and Keiji Wakabayashi[1]

[1]Cancer Prevention Basic Research Project, National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan, [2]Japan Food Research Laboratories, Bunkyo 2-3, Chitose-shi, Hokkaido 066-0052, Japan and [3]Minase Research Institute, Ono Pharmaceutical Co. Ltd., 1-1, Sakurai 3-chome, Shimamoto-cho, Mishima-gunn, Osaka 618-8585, Japan

*To whom correspondence should be addressed. Tel: +81 3 3542 2511; Fax: +81 3 3543 9305;
Email: mtakahas@ncc.go.jp

Elevated protein expression of inducible nitric oxide synthase (iNOS) has been observed in human pancreatic cancers and therefore, iNOS may play important roles in pancreatic carcinogenesis. This was examined in the present study, using an experimental model with *N*-nitrosobis(2-oxopropyl)amine (BOP)-treated hamsters. Reverse transcription–polymerase chain reaction analysis demonstrated iNOS expression in a hamster pancreatic cancer cell line as well as in human pancreatic cancer cell lines. Immunohistochemical analysis revealed increased expression of iNOS protein in atypical hyperplasia and ductal adenocarcinomas of the pancreas in BOP-treated hamsters. In addition, iNOS expression was also observed in macrophages and islet cells in pancreatic tissue surrounding tumors. In order to assess the role of iNOS expression in carcinogenesis in the pancreas, the effects of ONO-1714 [(1*S*, 5*S*, 6*R*, 7*R*)-7-chloro-3-imino-5-methyl-2-azabicyclo[4.1.0]heptane], an iNOS inhibitor, on hamster pancreatic ductal carcinogenesis were investigated. Female Syrian golden hamsters were treated with BOP at 10 mg/kg body wt, four times for 1 week, and 1 week after the last carcinogen treatment, ONO-1714 was administered at doses of 100 and 200 p.p.m. in the diet for 15 weeks. The incidences and multiplicities of atypical hyperplasia and invasive adenocarcinoma and total adenocarcinomas (non-invasive and invasive adenocarcinomas) in the pancreas were significantly lowered by treatment with 200 p.p.m. ONO-1714. Treatment with 100 p.p.m. ONO-1714 also significantly decreased the multiplicities of invasive and total adenocarcinomas. Moreover, treatment with 200 p.p.m. ONO-1714 reduced the number of BOP-induced cholangiocellular tumors. These results suggest that iNOS plays roles in promoting pancreatic carcinogenesis in both early and late stages in hamsters.

Introduction

Pancreatic cancer is steadily increasing in incidence and has a very poor prognosis (1). For development of effective chemotherapeutic and chemopreventive agents, elucidation of causative factors and mechanisms underlying pancreatic carcinogenesis is very important. As with other cancers, chronic inflammation is considered to be one of the risk factors (2). Epidemiological studies have shown that in addition to the environmental factors like cigarette smoking and dietary habits, pancreatitis is very important (3). Causes of pancreatitis are known to include alcohol drinking, smoking, gallstones, hyperlipidemia and stress (4,5).

Abbreviations: BOP, *N*-nitrosobis(2-oxopropyl)amine; cDNA, complementary DNA; iNOS, inducible nitric oxide synthase; IL, interleukin; NO, nitric oxide; NOS, nitric oxide synthase.

Chronic inflammation is associated with release of many cytokines and activation of nuclear factor κB, resulting in the expression of nuclear factor κB-regulated, inflammatory-related genes, such as inducible nitric oxide synthase (iNOS) (6). The resultant overproduction of nitric oxide (NO) contributes to multistage carcinogenesis by inducing DNA mutations and tissue damage (6). Increased expression of iNOS in human pancreatic cancers has been described (7–9) and expression has been also reported in a rat pancreatitis model (10).

Suppressive effects of iNOS-selective inhibitors, (1*S*, 5*S*, 6*R*, 7*R*)-7-chloro-3-imino-5-methyl-2-azabicyclo[4.1.0] heptane (ONO-1714) and L-N^6-(1-iminoethyl)lysine tetrazole-amide (SC-51), on pancreatitis in rats have been reported (11,12). ONO-1714 is 10-fold more selective for human iNOS than for human endothelial nitric oxide synthase (NOS), very potent in inhibiting plasma NO elevation in lipopolysaccharide-treated mice with a 50% inhibition dose of 0.010 mg/Kg subcutaneously and less toxic with a maximum tolerated dose of 30 mg/kg intravenously in mice (13,14). In addition, ONO-1714 is effective even when orally administered and our previous studies have demonstrated suppressive effects of ONO-1714 on growth of tumors formed in nude mice after subcutaneous injection of the K-*ras* mutant-transfected cells (15), aberrant crypt focus formation and large tumor development in the colon of rats treated with azoxymethane (16) and on colon cancer development in Min mice treated with dextran sodium sulfate (17). However, to our knowledge, there have hitherto been no reports concerning effects of iNOS inhibitors on pancreatic cancer development.

The Syrian golden hamster provides a unique model animal for the development of ductal pancreatic cancer. With subcutaneous injections of *N*-nitrosobis(2-oxopropyl)amine (BOP) (18), lesions having close similarities to the major form of pancreatic cancer in humans are induced. Point mutations in codon 12 of the K-*ras* gene are frequently observed (19), and expression of the *fragile histidine triad* gene, a tumor suppression gene, is generally abnormal in pancreatic cancers of hamsters (20), as in human tumors (21,22). Upregulation of cyclooxygenase-2 has been also observed in both BOP-induced pancreatic neoplastic lesions in hamsters and in human lesions (23), although there has been no report of iNOS expression in hamster pancreatic cancer. Therefore in the present study, we examined expression of iNOS in hamster pancreatic ductal cancer and investigated suppressive effects of ONO-1714, an iNOS-selective inhibitor, on hamster pancreatic ductal carcinogenesis induced by BOP.

Materials and methods

Chemicals

(1*S*, 5*S*, 6*R*, 7*R*)-7-chloro-3-imino-5-methyl-2-azabicyclo[4.1.0] heptane (ONO-1714) was chemically synthesized at Ono Pharmaceutical Co. Ltd (Osaka, Japan). BOP was obtained from Nacalai Tesque (Kyoto, Japan).

Cell culture

A hamster pancreatic cancer cell line, HaP-T1 (24), was obtained from RIKEN Cell Bank (Saitama, Japan) and a hamster pancreatic β-cell cell line, HIT-T15, from Dainippon Pharmaceutical Co., Ltd (Osaka, Japan). Human pancreatic cancer cell lines, Capan-2, HPAF-II, HPAC, Hs-776T, MiaPaca-2 and Panc-1, were obtained from Summit Pharmaceutical International Co., LTD (Tokyo, Japan) and BxPC-3 from RIKEN Cell Bank. A human pancreatic normal ductal cell line, HPDE-6 (25), was kindly provided by Dr Ming-Sound Tsao (University Health Network, Toronto, Ontario, Canada). The cells were maintained in RPMI-1640 (Iwaki, Japan), supplemented with 5% fetal bovine serum (HyClone Laboratories, Logan, UT) and 100 units/ml penicillin–streptomycin (GIBCO/Invitrogen Corp., Carlsbad, CA) at 37°C in 5% CO_2. To induce iNOS expression, cells were treated with 10 ng/ml of mouse or human interleukin (IL)-1β (Sigma Chemical Co., St Louis, MO) for 6 h.

Animals

Five-week-old female Syrian golden hamsters weighing ~80 g were obtained from Japan SLC (Shizuoka, Japan) and acclimated to laboratory conditions for a week. They were housed two or three per plastic cage, with sterilized soft-wood chips as bedding, in an air-conditioned animal room, on a 12 h light–dark cycle. Powdered CE-2 (CLEA Japan, Shizuoka, Japan) was used as a standard basal diet. Body weights were measured on a weekly basis and food consumption twice a week. Food and water were made available *ad libitum*.

Reverse transcription–polymerase chain reaction analysis

Total RNA was extracted from culture cell samples using ISOGEN (Wako Pure Chemical Industries, Ltd, Osaka, Japan). After RNA purification, aliquots of total RNA (2 μg) were subjected to the reverse transcription reaction with oligo-dT or 9mer random primers in a final volume of 20 μl using an Omuniscript Reverse Transcription Kit (Qiagen GmbH, Heilden, Germany). Polymerase chain reaction amplification was performed in a final volume of 10 μl with aliquots of complementary DNA (cDNA) (25 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 sequences of β-actin (26) and the common sequences between hamster and human cDNA of iNOS (accession numbers, AY297461 and D26525)—5′-primer: ACGAGGCCCAGAGCAAGAGA, 3′-primer: TGGCTGGGGTGTTGAAGGTC (product size, 228 bp) for β-actin and 5′-primer: TTCCCCCAGCGGAGTGATGG, 3′-primer: GTACCAGCCATTGAAGGGGC (product size, 382 bp) for iNOS. The cycling conditions were as follows: 95°C for 3 min, 26 cycles (for β-actin) or 35 cycles (for iNOS) of 94°C for 15 s, 60°C for 25 s and 72°C for 30 s and a 10 min cycle at 72°C. Products were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining.

Immunohistochemistry

Paraffin sections from formalin-fixed tissues of hamster normal pancreas and pancreatic tumors obtained in our previous study (26) were used for immunohistochemical analyses with the avidin–biotin complex immunoperoxidase technique as described previously (27). As the primary antibody, monoclonal mouse anti-iNOS IgG (BD Biosciences Pharmingen, San Diego, CA #610328) was applied at 50× dilution. As the secondary antibody, biotinylated anti-mouse IgG raised in a horse, affinity purified and absorbed with rat serum (Vector Laboratories, Burlingame, CA) was employed at 200× dilution. Staining was performed using avidin–biotin reagents (Vectastain ABC reagents; Vector Laboratories), 3,3′-diaminobenzidine and hydrogen peroxide. The sections were counterstained with hematoxylin. As a negative control, duplicate sections were immunostained without exposure to the primary antibody.

Study on the effects of ONO-1714, an iNOS inhibitor, on BOP-induced pancreatic carcinogenesis in hamsters

A total of 126 hamsters at 6 weeks of age were injected subcutaneously with BOP four times (on days 1, 3, 5 and 7) at a dose of 10 mg/kg body wt, whereas 18 hamsters received saline as vehicle controls. One week after the last BOP treatment, one-third of each group was given basal diet, diet containing 100 p.p.m. or 200 p.p.m. of ONO-1714 for 15 weeks. The doses were based on our previous study in mice (15,17) and rats (16) and a preliminary study in hamsters (data not shown). At the killing time point at 23 weeks of age, all surviving animals were anesthetized with diethyl ether, and blood samples were collected from the aorta. 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 paraffin-embedded organs were sectioned and stained with hematoxylin and eosin for assessment of histopathological features, as described previously (26). The experimental protocol was in accordance with the guidelines for Animal Experiments in the National Cancer Center and was approved by the Institutional Ethics Review Committee for Animal Experimentation.

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

iNOS expression in human and hamster pancreatic cancer cell lines

Expression of iNOS messenger RNA in human and hamster pancreatic cancer cell lines was examined by reverse transcription–polymerase chain reaction. As shown in Figure 1, iNOS was constitutively expressed in five of seven human pancreatic cancer cell lines, BxPC-3, HPAF-II, Hs-766T, MiaPaca-2 and Panc-1. On treatment with IL-1β, expression of iNOS in the other two cancer cell lines, Capan-2 and HPAC, and a human pancreatic normal ductal cell line, HPDE-6, was induced, and the expression in HPAF-II and Hs-766T was enhanced. Expression of iNOS was also constitutively observed in a hamster pancreatic cancer cell line, HaP-T1, and a hamster β-cell cell line, HIT-T15, and in both cases was markedly enhanced by treatment with IL-1β.

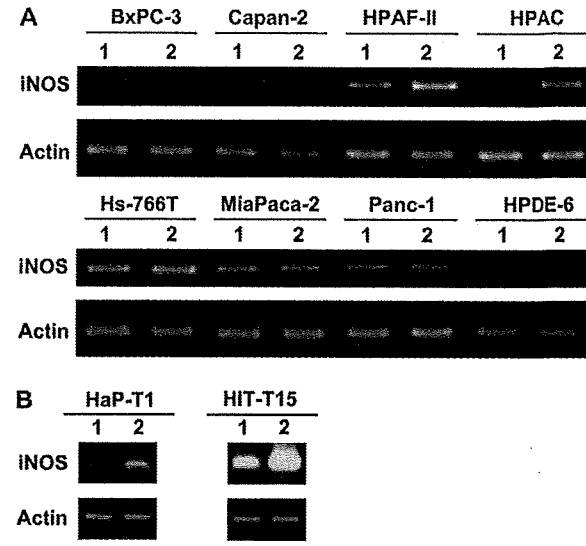

Fig. 1. Reverse transcription–polymerase chain reaction analysis of iNOS expression in human (**A**) and hamster (**B**) pancreatic cell lines. Pancreatic cancer cell lines (BxPC-3, Capan-2, HPAF-II, HPAC, Hs-766T, MiaPaca-2, Panc-1 and HaP-T1), a normal pancreatic ductal epithelial cell line (HPDE-6) and an islet β-cell line (HIT-T15) were incubated in basal medium (lane 1) or treated with 10 ng/ml IL-1β (lane 2) for 6 h. Total RNA from each sample was extracted and cDNA was synthesized by reverse transcription. Then, cDNA fragments of iNOS and β-actin were amplified by polymerase chain reaction, and the polymerase chain reaction products were electrophoresed on a 2% agarose gel.

iNOS expression in hamster pancreatic ductal adenocarcinomas induced by BOP

Expression of iNOS protein in hamster pancreatic ductal cancers was examined by immunohistochemical staining. In normal pancreatic tissue, expression of iNOS protein was barely detectable in non-treated normal pancreatic tissue (Figure 2A) and non-tumorous parts of BOP-treated pancreatic tissue (Figure 2B). In contrast, positive staining for iNOS was clearly observed in macrophages and islet cells in areas of inflammation, atypical hyperplasia in ducts (Figure 2C) and in the carcinoma epithelial cells (Figure 2D). All 12 pancreatic carcinomas examined were positive for iNOS staining: six demonstrated strong, four moderate and two weak staining in the cytoplasm of epithelial cancer cells.

Effects of ONO-1714 on pancreatic tumor development in BOP-treated hamsters

To examine the role of iNOS on pancreatic carcinogenesis, hamsters were treated with a pancreatic carcinogen, BOP, then were fed a diet containing the iNOS inhibitor, ONO-1714, at doses of 100 or 200 p.p.m. for 15 weeks. The final body weights (g) and average food intake of hamsters are shown in Table I. The body weights and average food intake in the BOP + basal diet group were lower than those in the saline + basal diet group ($P < 0.05$), and the average body weight in the saline + 200 p.p.m. ONO-1714 group was 13% lower