Original Article

Sesamol suppresses cyclooxygenase-2 transcriptional activity in colon cancer cells and modifies intestinal polyp development in *Apc*$^{Min/+}$ mice

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Excessive prostaglandin production by cyclooxygenase-2 in stromal and epithelial cells is a causative factor of colorectal carcinogenesis. Thus, compounds which inhibit cyclooxygenase-2 transcriptional activity in colon epithelial cells could be candidates for anti-carcinogenic agents. A cyclooxygenase-2 transcriptional activity in the human colon cancer cell line DLD-1 has been measured using a β-galactosidase reporter gene system. Using this system, we demonstrated that the decrease in basal cyclooxygenase-2 transcriptional activities at 100 μM sesamol, one of the lignans in sesame seeds, was 50%. Other compounds in sesame seeds such as sesamin, sesamolin, ferulic acid, and syringic acid did not exhibit significant suppression of cyclooxygenase-2 transcriptional activity at up to 100 μM. In a following experiment, 6-week-old male *Min* mice, *Apc*-deficient mice, were divided into a non-treated and 500 ppm sesamol groups. At the age of 15 weeks, it was found that treatment with sesamol decreased the number of polyps in the middle part of small intestine to 66.1% of the untreated value. Moreover, sesamol suppressed cyclooxygenase-2 and cytosolic prostaglandin E_2 synthase mRNA in the polyp parts. The present findings may demonstrate the novel anti-carcinogenetic property of sesamol, and imply that agents that can suppress cyclooxygenase-2 expression may be useful cancer chemopreventive agents.

Key Words: **cyclooxygenase-2, reporter gene assay, sesame, sesamol, *Min* mice**

The sesame plant (*Sesamun indicum*, Linn.) is well known for its edible seeds and oil.[1] Sesame seeds are characterized by the presence of fatty acids (linoleic acid, linolenic acid, oleic acid, palmitic acid and stearic acid), oil-soluble lignans (episesamin, sesamin, sesaminol, sesamol and sesamolin) and other phenol compounds (γ-tocophenol, ferulic acid and syringic acid). The nonfat portion of sesame seed is only 1–2% by wet weight. Recently, multiple biological functions of sesame seeds, such as inhibition of inflammation and carcinogenesis, have been elucidated. In experimental studies, sesamol was shown to inhibit development of spontaneous development of preneoplastic hepatocytic foci in rats.[2] Sesamin reduced the incidence of chemically induced rat mammary gland cancers.[3] Moreover, sesame oil has been reported to inhibit growth of human colon cancer cells *in vitro*.[4] These effects of sesame seed and its constituents were partly associated with its hydroxyl radical scavenging activity, inhibitory activity of lipid peroxidation and anti-mutageneic activity.[5–7] However, the desirable biological functions have not entirely been elucidated yet.

Recent accumulating evidence has indicated that prostaglandins (PGs) are implicated in colon carcinogenesis.[8] Expression levels of cyclooxygenase-2 (COX-2) are increased in colon carcinoma tissues compared to that of normal colonic mucosa. Therefore, inhibitors against COX-2 have been studied extensively for their ability to suppress colon carcinogenesis. It has been also reported that COX-2 gene knockout causes significant reduction in number and size of intestinal polyps in a mouse model for human familial adenomatous polyposis, *Apc*-deficient *Min* mice.[9] Thus, it is likely that agents that can suppress COX-2 expression at the transcriptional level may be equally advantageous.

As reported in previous papers,[10–12] we have constructed a β-galactosidase reporter gene system to test the effects of compounds on COX-2 transcriptional activity in a human colon cancer cell line, DLD-1 cells. In the present study, effects of five sesame seeds constituents on the transcriptional activity of COX-2 were investigated and one constituent, sesamol, was found to suppress basal COX-2 transcriptional activity. In a further experiment, we investigated the suppressive effect of sesamol on intestinal polyp development and on COX-2 expression levels in *Min* mice.

Materials and Methods

Chemicals. Sesamin were obtained from Cayman Chemical, (Ann Arbor, MI), ferulic acid, sesamol and syringic acid were from Sigma-Aldrich Co. (St. Louis, MO). Sesamolin was from Nagara, Ltd. (Gifu, Japan).

Cell culture. DLD-1 cells, a human colon adenocarcinoma cell line, were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Construction of DLD-1/COX-2-B2-βGal-BSD cells has been reported in our previous papers.[13] The cells were maintained in DMEM medium supplemented with 5% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT) and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin) at 37°C in 5% CO_2.

Measurements of cell viability. Cell viability in each culture was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells at a density of 2.0×10^4 cells per well were seeded in 96-well tissue culture plates

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and treated with sesame constituents for 48 h. After treatment, the cells were further incubated in a medium containing 0.5 mg/mL of MTT for 1 h. The MTT formazan produced by living cells was dissolved in dimethyl sulfoxide and absorbance at 595 nm was measured on a microplate Reader (Bio-Rad Laboratories, CA).

Reporter gene assay for COX-2 promoter-dependent transcriptional activity. DLD-1/COX-2-B2-βGal-BSD cells were seeded at a density of 2.0×10^4 cells per 96-well tissue culture plate and precultured for 24 h. After treatment with the test reagents, the total β-galactosidase activities of the cells in each well were determined by colorimetric assay using *o*-nitrophenyl-β-d-galactopyranoside (ONPG) as described previously.[10,11] The background β-galactosidase activity of DLD-1 cells was determined in non-treated DLD-1/B2-βGal-BSD cells, and the value was set as 0. Basal β-galactosidase activity of non-treated DLD-1/COX-2-B2-βGal-BSD cells was set as 100%. The percent β-galactosidase activity of each treatment was calculated from triplicate wells. The viable cell number was assessed by the MTT assay. All assays, including MTT assay, were carried out in triplicate and each experiment repeated at least three times.

Quantitative real-time polymerase chain reaction (PCR) analysis. Total RNA was isolated using TRIzol Reagent (Invitrogen, NY), treated with DNase (Invitrogen, Grand Island, NY) and 1 μg aliquots in a final volume of 20 μL were used for synthesis of cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was carried out using Fast Start Universal SYBR Green Mix (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Primers for COX-1 (5'-TGA TGC TCT TCT CCA CGA and 5'-GCA GGA AAT AGC CAC TCA AG), COX-2 (5'-GTG CCA ATT GCT GTA CAA GC and 5'-TAC AGC TCA GTT GAA CGC CT), cPGES (5'-AGT CAT GGC CTA GGT TAA C and 5'-TGT GAA TCA TCA TCT GCT CC), EP1 (5'-ACC CTG CAT CCT GAG CAG CAC TGG CCC TCT and 5'-CGA TGG CCA ACA CCA CCA ACA CCA GCA GGG), EP2 (5'-AGG ACT TCG ATG GCA GAG GAG AC and 5'-CAG CCC CTT ACA CTT CTC CAA TG), EP3 (5'-TGA CCT TTG CCT GCA ACC TG and 5'-AGA CAA TGA GAT GGC CTG CC), EP4 (5'-TCC CGC TCG TGG TGC GAG TGT TC and 5'-GAG GTG GTG TCT GCT TGG GTC AG), mPGES-2 (5'-AAG ACA TGT CCC TTC TGC and 5'-CCA AGA TGG GCA CTT TCC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5'-TGT CAG CAA TGC ATC CTG CA and 5'-TTA CTC CTT GGA GGC CAT GT) were employed. For evaluation of human mRNA levels, indicated primers as shown below were used. Human EP1 (5'-TCT ACC TCC CTG CAG CGG CCA CTG and 5'-GAA GTG GCT GAG GCC GCT GTG CCG GGA), human EP2 (5'-ATG GGC AAT GCC TCC AAT GAC TCC CAG and 5'-CTC CAG GGA ACA ATT TCA AAA T), human EP4 (5'-CCT CCT GAG AAA GAC ACT GCT and 5'-AAG ACA CTC TCT GAG TCC T), and human GAPDH (5'-CCA CCC ATG GCA AAT TCC and 5'-TGG GAT TTC CAT TGA TGA CAA). To assess the specificity of each primer set, amplicons generated from the PCR reaction were analyzed for melting curves.

Western blot analysis. EP1–4 protein levels were analyzed by western blot. DLD-1/COX-2-B2-βGal-BSD cells were seeded at a density of 2×10^5/well in 24-well plates, and incubated with 50 and 100 μM sesamol for 24 and 48 h. 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]. Samples were separated in 10% polyacrylamide gel electrophoresis–sodium dodecyl sulfate gels and transferred onto polyvinylidene difluoride membranes (Millipore, MA). Abs against the EP1, EP2, EP4 (Cayman Chemical Co. Ann Arbor, MI) and EP3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at a 1:2,000 dilution. Peroxidase-conjugated secondary Abs for anti-rabbit IgG were obtained from GE Healthcare (Buckingham shire, UK). Blots were developed with ECL western blotting detection reagents (GE Healthcare).

Animals. Female C57BL/6-*Apc*$^{Min/+}$ mice (*Min* mice) were purchased from The Jackson Laboratory (ME). 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. Sesamol was well mixed at a concentration of 500 ppm in AIN-76A powdered basal diet (CLEA Japan, Tokyo, Japan).

Protocol for Animal experiments. Ten female *Min* mice at 5 weeks of age were given 500 ppm sesamol, for 8 weeks. The animals in each cage were all in the same treatment group. 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. The intestinal tract was removed 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. Polyps in the proximal segments were counted and all

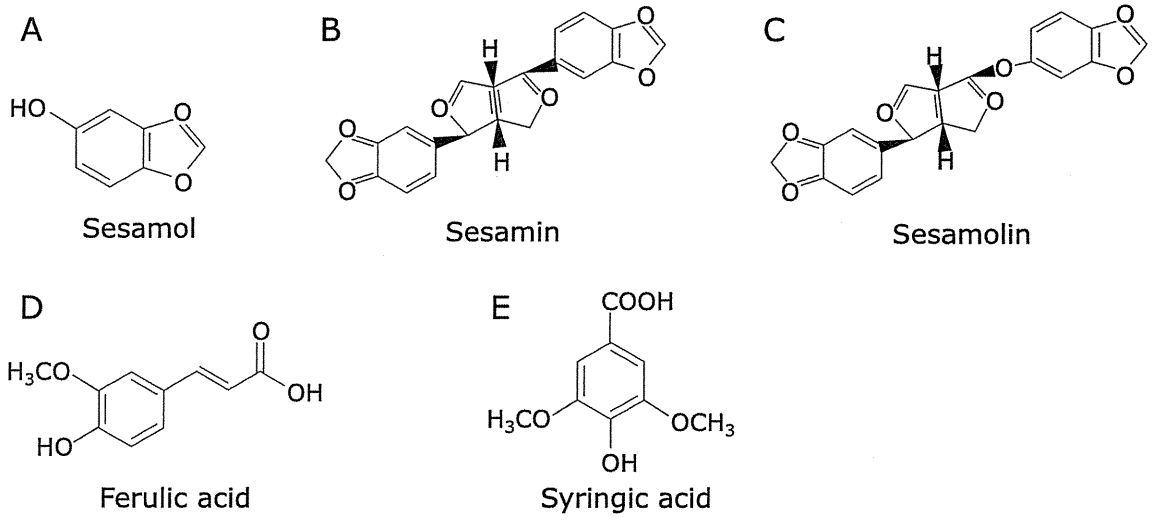

Fig. 1. Chemical structures of the five constituents in the sesame seed. (A), sesamol; (B), sesamin; (C), sesamolin; (D), ferulic acid; (E), syringic acid.

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polyps in the proximal segment were picked up under a stereoscopic microscope and the remaining intestinal mucosa (non-polyp part) was removed by scraping, and then both stored at –80°C for further analysis. Other 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. The experiments were performed according to the "Guidelines for Animal Experiments in the National Cancer Center" and were approved by the Institutional Ethics Review Committee for Animal Experimentation in the National Cancer Center.

Statistical analysis. All the results are expressed as mean ± SD. values, with statistical analysis using the Student's *t* test, except for the COX-2 promoter activity investigation and mRNA examination in the human cell line. The Bonferroni *z* test was used for statistical analyses of the COX-2 promoter activity and of human mRNA levels. Differences were considered to be statistically significant at $p<0.05$.

Results

Suppression of COX-2 promoter activity in human colon cancer cells by sesamol. Five compounds, shown in Fig. 1, were tested at various concentrations up to 100 μM with regard to their effects on COX-2 promoter activity. Remarkable suppression of cell proliferation rates by five compounds was not observed at the concentrations up to 100 μM in MTT assay. Among five constituents in the sesame seed, only sesamol significantly suppressed COX-2 prompter activity in a dose-dependent manner. Decrease in COX-2 promoter activities by sesamol at 100 μM was 50% (Fig. 2A). The other four compounds, ferulic acid, sesamin, sesamolin, syringic acid exhibited weak or no suppression of COX-2 promoter activity (Fig. 2B–E).

Suppression of intestinal polyp formation in *Min* mice by sesamol. Administration of 500 ppm sesamol to *Min* mice for 8 weeks did not affect body weights, food intake or clinical signs throughout the experimental period. Average daily food intake did not significantly differ among the groups, being 3.9 and 3.5 g per mouse per day for the 0 and 500 ppm group of *Min* mice, respectively. In addition, there were no changes observed in any organ weights that might have been attributable to toxicity.

Table 1 summarizes data for the number and distribution of intestinal polyps in the basal diet and sesamol-treated groups. Almost all polyps developed in the small intestine, with only a few in the colon. The total number of polyps tended to be decreased by administration of 500 ppm sesamol to 75% of the untreated control value. Reduction of polyps was observed in the middle part, and was by 66% ($p<0.05$ vs 0 ppm). In the other parts of small intestine and colon, treatment with sesamol lowered the number of polyps without significant difference.

Fig. 3 shows the size distribution of intestinal polyps in the basal diet and sesamol-treated groups. The maximal number of polyps was observed in the size range between 0.5 and 2.0 mm in diameter. Administration of sesamol significantly reduced the numbers of polyps sized <0.5 mm in diameter.

Decrease of inflammation-related factors mRNA levels in intestinal polyp parts by sesamol. Inflammation-related factors mRNA expressions in intestinal polyp parts and non-polyp parts were investigated (Fig. 4). Real-time PCR revealed that treatment with 500 ppm sesamol for 8 weeks significantly suppressed COX-2, and cPGES, mRNA levels in the intestinal polyp parts to 48% ($p<0.01$) and 54% ($p<0.05$) of sample value, respec-

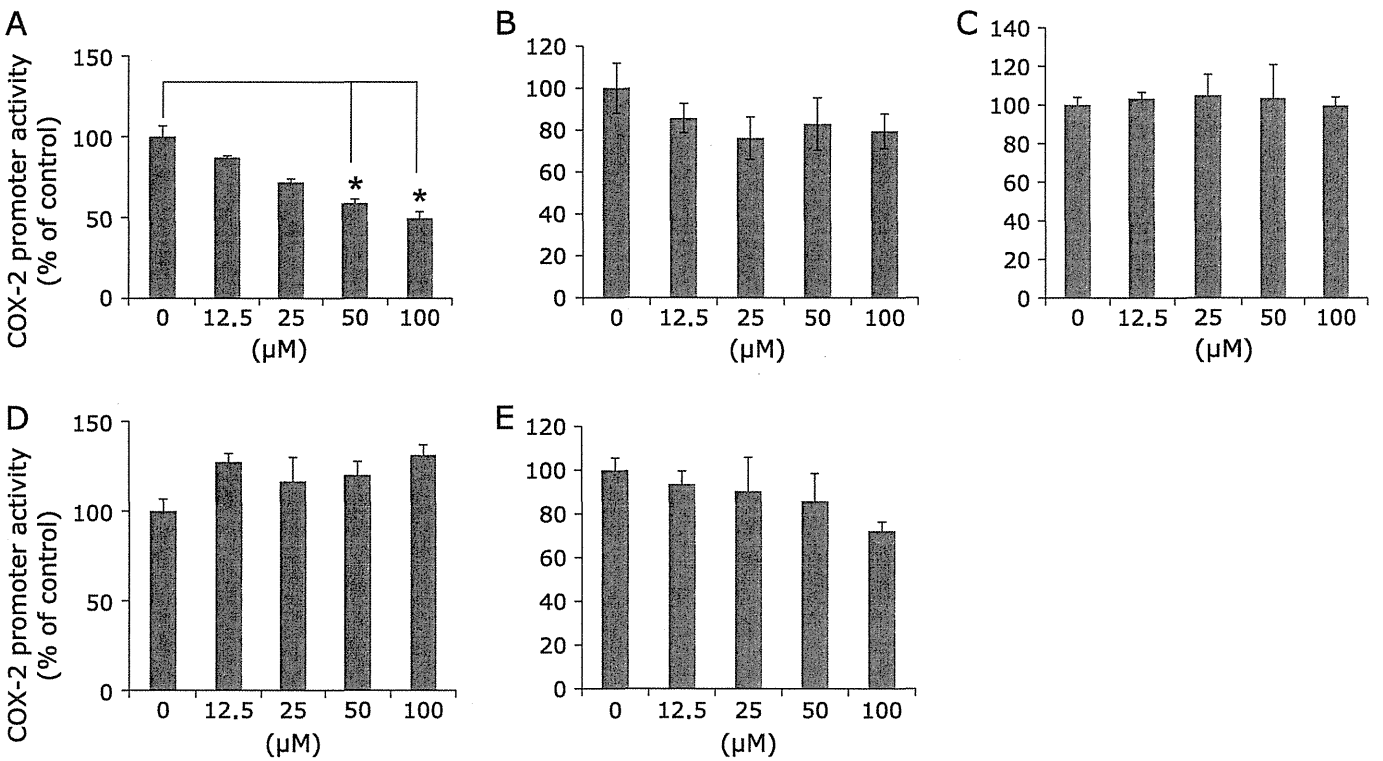

Fig. 2. Effects of treatment with sesame constituents on reporter gene activity in DLD-1/COX-2-B2-β-gal-BSD cells. DLD-1/COX-2-B2-β-gal-BSD cells were seeded in 96-well multiwell plates at a density of 2×10^5 cell/well and cultured in medium containing sesame constituents at concentrations up to 100 μM. After 48 h, the COX-2 promoter activity was evaluated by β-galactosidase activity and was normalized for viable cell numbers assessed by MTT assay. The columns indicate the values of the mean percentages of triplicate wells of promoter activity of DLD-1/COX-2-B2-β-gal-BSD cells. The data are representative of more than three independent experiments. Bars indicate the SD. *$p<0.05$. (A), sesamol; (B), sesamin; (C), sesamolin; (D), ferulic acid; (E), syringic acid. COX; cyclooxygenase, cPGES; cytosolic PGES, mPGES; microsomal PGES, PGES; prostaglandin E synthase.

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Table 1. Number of intestinal polyps/mouse in *Min* mice

Sesamol (ppm)	No. of mice	Small intestine			Colon	Total
		Proximal	Middle	Distal		
0	9	4.9 ± 4.6	17.1 ± 5.6	22.1 ± 11.7	0.7 ± 1.3	44.8 ± 15
500	8	2.4 ± 1.5	11.3 ± 5.6*	19.9 ± 4.4	0.1 ± 0.4	33.6 ± 9.2#

Data are mean ± SD. *Significantly different from the control untreated group at $p<0.01$. #$p = 0.087$.

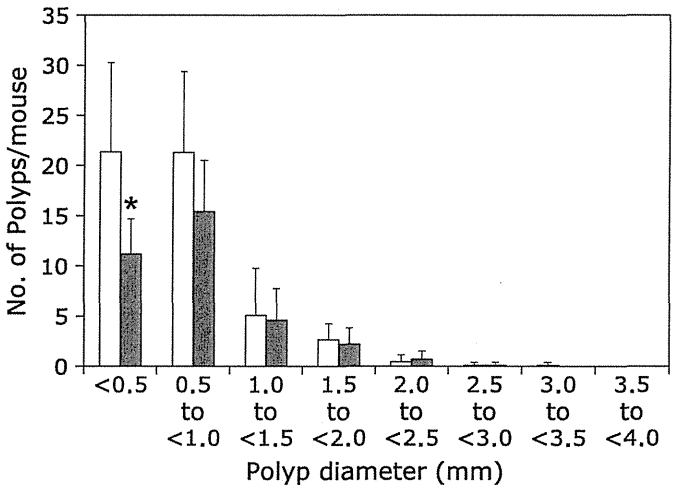

Fig. 3. Effects of sesamol on the size distribution of intestinal polyps in *Min* mice. *Min* mice were fed a basal diet (open box) or a containing 500 ppm (black filled box) sesamol for 8 weeks. The number of polyps per mouse in each size class is given as a mean ± SD. *$p<0.05$.

tively. mPGES2, EP1 and EP2 mRNA levels tended to be reduced in intestinal polyp parts by sesamol. COX-1 and EP4 mRNA expression levels in non-polyp parts and polyp parts of small intestine were not suppressed by sesamol treatment. Only EP3 did not increase its expression levels in the intestinal polyp parts compared to those of mucosa parts, and rather decreased its expression by sesamol treatment in the intestinal polyp parts.

Decrease of prostaglandin E_2 receptor expression levels in human colon cancer cells by sesamol. To clarify the effects of sesamol on EP1 and EP2 mRNA levels in human cells, we treated DLD-1/COX-2-B2-β-gal-BSD cells with 50 and 100 μM sesamol for 48 h. Sesamol significantly suppressed EP1 and EP2 mRNA levels in a dose-dependent manner (Fig. 5). However, mRNA levels for EP4 were increased and EP3 was not clearly detected (data not shown). We further confirmed the EP1–4 protein expression levels in the cells with or without sesamol treatment. As shown in Fig. 5D, sesamol down-regulated EP1 and EP2 protein levels in dose- and time-dependent manner. As in the case of EP4, sesamol treatment for 24 h slightly increased its protein levels, whereas 48 h treatment decreased EP4 protein levels. EP3 expression levels seem to be very low in these cells, as shown in Fig. 5D.

Discussion

In the present study, sesamol was found to suppress basal transcriptional activity of the COX-2 gene in human colon cancer DLD-1 cells. Previously, we have reported that mono-benzonic compounds such as resorcinol and resacetophenone suppress COX-2 transcriptional activity,[10,11] but their inhibitory activities are almost 5-times less than that of sesamol. Thus, sesamol may have a notable potential to suppress COX-2 expression for a natural compound.

The underlying mechanism of suppression of COX-2 transcriptional activity by sesamol is not clear. Protein-tyrosine kinases (PTKs), including the epidermal growth factor receptor, are well known to be involved in the induction of COX-2 expression.[14,15] Signals from activated PTKs are transduced to the downstream transcription factor NF-κB, mainly by the Ras and mitogen-activated protein kinase pathways. It is also known that activation of NF-κB has been reported to play an important role in the regulation of COX-2 expression. However, our preliminary experiment that aimed to evaluate the effects of sesamol on NF-κB transcriptional activity failed to show its reduction at concentrations up to 100 μM in human colon cancer cells (data not shown). Further studies are needed to elucidate the molecular mechanisms responsible for the inhibition of COX-2 transcriptional activity by sesamol.

We next aimed to show the suppressive potential of sesamol on intestinal polyp development in *Min* mice. Administration of 500 ppm sesamol tended to reduce the total number of intestinal polyps development compared to that of the untreated group. Further analysis revealed that treatment with sesamol decreased the number of polyps in the middle part of the small intestine. It has been reported that indomethacin, a COX inhibitor, and nimesulide, a COX-2 selective inhibitor, mainly reduce the number of polyps in the middle to distal part of the small intestine.[16,17] Thus, sesamol with a COX-2 suppressive function has a similar inhibitory potential for polyp development. For instance, LPL inducers such as NO-1886 or PPAR ligands effectively reduce the number of polyps in the proximal part of the small intestine.[18,19]

In the polyp parts of *Min* mice, it was confirmed that sesamol could suppress expression levels of COX-2 mRNA. In addition, cPGES mRNA was reduced by sesamol treatment, and this is the first report that suggests suppressive effects of sesamol on cPGES as far as we know. Moreover, a tendency to suppression was observed in the expression levels of PGE_2 receptor subtypes EP1 and EP2 in the polyp parts of *Min* mice. Using PGE_2 receptor subtype-knockout mice, the roles of these receptors in colon carcinogenesis have been investigated.[20–23] These observations suggest that EP1, EP2 and EP4 are promotive receptors in colorectal carcinogenesis, and EP3 plays suppressive roles. EP1 signals transmitted by increased intracellular Ca^{2+} concentrations activate protein kinase C (PKC). However, the actual signal transduction mechanisms are not known in detail.[24,25] Stimulation of EP2 and EP4 receptors in both cases involves coupling with stimulatory G protein, leading to activation of adenylate cyclase. As a result, increased cAMP levels activate cAMP-dependent protein kinase (PKA) and increase a transcriptional factor that binds to cAMP-responsive element, that plays a role in cell growth and cell survival. Thus, it may be worthwhile to develop functional inhibitors or specific suppressors for EP1, EP2 and EP4. However, it is regrettable that there are a few inhibitors for PGE_2 receptor subtypes. To add to the novel potential of sesamol, we confirmed the effect of sesamol on human EP1 and EP2 mRNA levels. We found suppression of EP1 and EP2 mRNA levels by sesamol treatment. Down-regulation of EP1 and EP2 protein was also confirmed. These data imply a double suppressive potential exists in sesamol regarding cell growth function of PGE_2. Suppression of COX-2 may reduce production of growth lipid mediator PGE_2, and down regulation of PGE_2 receptors such as

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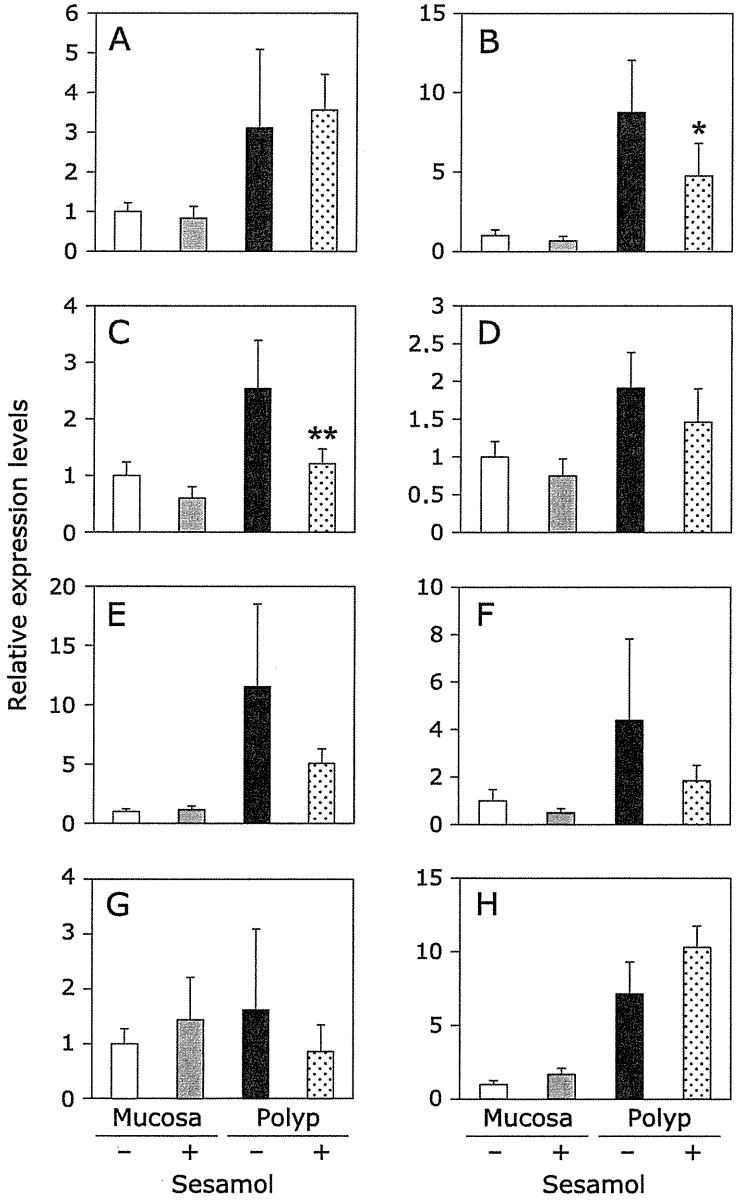

Fig. 4. Changes of inflammation-related factors in intestinal non-polyp mucosa parts and/or polyp parts of Min mice. Quantitative real-time PCR analysis were performed to determine COX-1 (A), COX-2 (B), cPGES (C), mPGES2 (D), EP1 (E), EP2 (F), EP3 (G), EP4 (H) mRNA expression levels in the polyps or non-polyp mucosa parts of *Min* mice, given diets containing sesamol at doses of 500 ppm for 8 weeks. Data are normalized with GAPDH expression level. Data are mean ± SD, $n = 6$. $^{**}p<0.01$, $^{*}p<0.05$ vs 0 ppm.

EP1 and EP2 may additionally suppress tumor growth through a transmembrane G protein-coupled receptor.

In summary, sesamol suppressed the transcriptional activity of COX-2 gene in DLD-1 cells. Moreover, our *in vivo* data imply that agents that can suppress COX-2 expression at the gene level may be useful cancer chemopreventive agents. Further information of the mechanisms by which sesamol suppresses COX-2 expression may clarify the anti-inflammatory and anti-carcinogenetic properties of sesamol.

Acknowledgments

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Abbreviations

cPGES	cytosolic PGES
COX	cyclooxygenase
FBS	fetal bovine serum
mPGES	microsomal PGES
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	nuclear factor-κB
ONPG	*o*-nitrophenyl-β-D-galactopyranoside
PGs	prostaglandins
PGES	prostaglandin E synthase
PTKs	protein-tyrosine kinases

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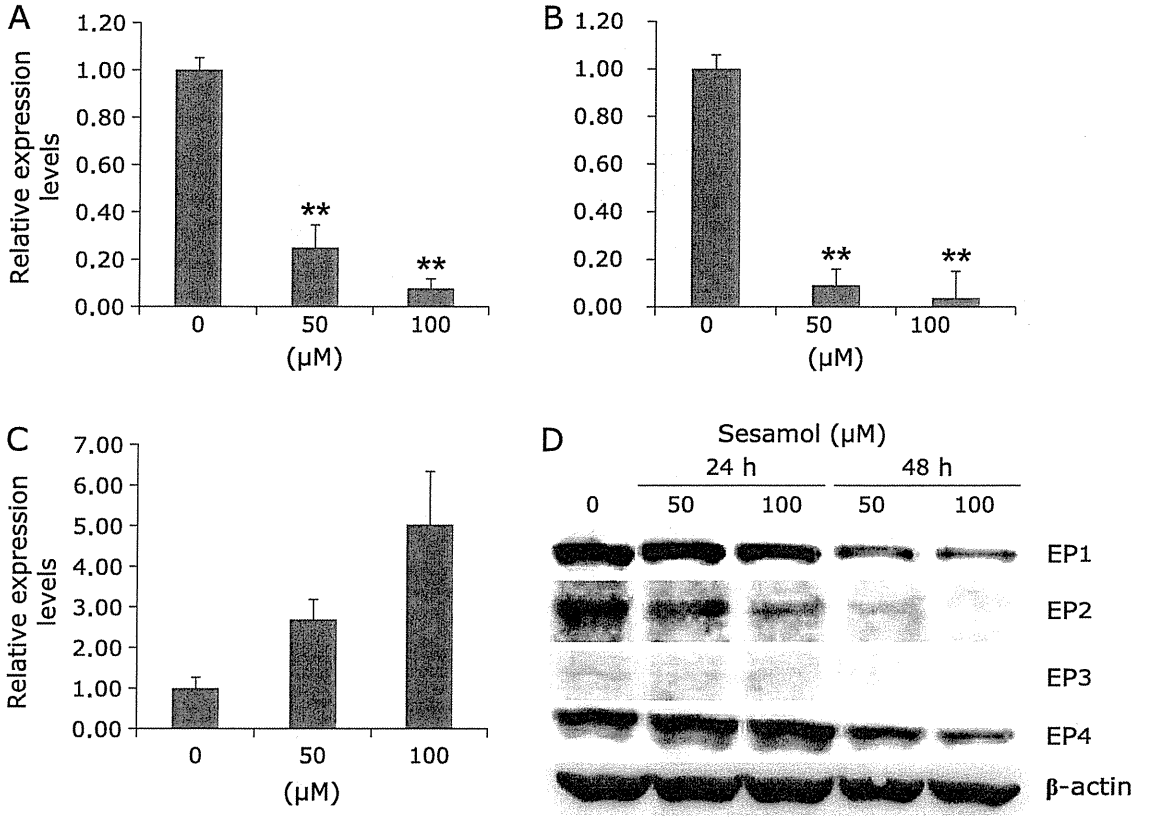

Fig. 5. Prostaglandin E_2 receptor expression in human colorectal cancer cells with or without sesamol treatment. DLD-1/COX-2-B2-β-gal-BSD cells were seeded in 6-well multiwell plates at a density of 2×10^6 cell/well and cultured in medium containing 50 and 100 μM sesamol for 48 h. After 48 h treatment, quantitative real-time PCR analysis was performed to determine EP1(A), EP2(B), EP4(C) mRNA levels. Data are normalized with GAPDH, Data are mean ± SD, $n = 3$.**$p<0.001$ vs 0 ppm. (D) EP1–4 protein was detected by western blot analysis, using DLD-1/COX-2-B2-β-gal-BSD cells (24-well plates at a density of 2×10^5 cell/well) with treatment of 50 and 100 μM sesamol for 24 and 48 h.

Conflict of Interest

No potential conflicts of interest were disclosed.

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Bi-directional Regulation Between Adiponectin and Plasminogen Activator-inhibitor-1 in 3T3-L1 Cells

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Abstract. *Background: Adiponectin (APN) and plasminogen activator inhibitor-1 (Pai-1) are adipocytokines, and low levels of serum APN and high levels of PAI-1 are observed in obese patients. Moreover, both APN and Pai-1 are known to be involved in colorectal carcinogenesis. Recently, we demonstrated that serum Pai-1 levels are elevated in APN-deficient mice. We hypothesized that Pai-1 expression levels could be depressed by APN. Thus, we aimed to clarify the bi-directional regulatory mechanisms between APN and Pai-1. Materials and Methods: We investigated the expression levels of APN and Pai-1 during 3T3-L1 pre-adipocyte differentiation, and examined the role of AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor (PPAR)-γ on APN and Pai-1 expression at early and late differentiation stages. Results: In the early phase of differentiation, Pai-1 expression increased and APN slightly decreased. Reduction of Pai-1 or activation of PPARγ resulted in elevation of APN, and supplementation of APN with activation of AMPK resulted in reduction of Pai-1. In the late phase of differentiation, APN increased its expression and Pai-1 decreased. Supplementation of Pai-1 resulted in a slight reduction of APN. Conclusion: It is suggested that APN and Pai-1 expressions are inversely-regulated. Understanding of the regulatory system between APN and Pai-1 may lead to finding novel methods for colorectal cancer prevention.*

Adiponectin (APN; 30 kDa protein) is one of the adipocytokines discovered in adipose tissue (1), and abundant amounts of APN are detected in plasma (3-30 μg/ml). A decrease of APN levels is associated with insulin-resistant type-2 diabetes, coronary artery disease, and the development of cancer, including colorectal cancer (2-6). There are two APN receptors, AdipoR1 and AdipoR2 (7). The physiological function of APN is evoked by binding to these receptors. It is known that AdipoR1 activates AMP-activated protein kinase (AMPK) and AdipoR2 activates peroxisome proliferator-activated receptor-α (PPARα) (8, 9).

We have been studying the involvement of APN in colorectal cancer risk. *Adenomatous Polyposis Coli* (*Apc*)-deficient Min mice ($Apc^{Min/+}$), a model of familial adenomatous polyposis (FAP), with APN deficiency were used to investigate the effects of *APN* knockout on intestinal polyp development. APN-deficient *Min* mice show a 2- or 3-fold increase in the total number of intestinal polyps developed compared with APN wild-type *Min* mice, regardless of gender (10). APN-deficient C57BL/6J mice treated with azoxymethane (AOM) demonstrated increased incidence and multiplicity of colorectal tumors, including adenomas and adenocarcinomas. *Min* mice exhibited an increase in serum plasminogen activator inhibitor-1 (Pai-1) levels with decreasing expression levels of *APN*. In addition, the tendency for elevation of serum Pai-1 levels was observed with APN-deficiency in C57BL/6J mice at the age of 55 weeks (10).

Pai-1 is one of the adipocytokines whose levels increase with obesity. Pai-1 directly inhibits tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). tPA and uPA activate plasminogen to produce plasmin through serine protease activity, and physiologically breakdown blood clots. Pai-1 is also reported to possess/exhibit multifunctional factors. Although the molecular mechanisms are not fully-established, Pai-1 was found to modulate cell proliferation and stimulate angiogenesis (11, 12). PAI-1 is known to be induced by triglyceride (TG), very low-density lipoprotein (TG-rich lipoprotein), transforming growth factor-β (TGFβ), various

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growth factors, tumor suppressor p53, nuclear factor kappa B (NFκB) and Wnt signaling (13-17), all of which are plausibly involved in carcinogenesis.

Adipocytokines can affect each other. Among them, APN is known to act as a major regulator of other adipocytokines. For instance, APN stimulates AMPK in the hypothalamus to promote food intake under starvation conditions and inhibit leptin activation (18). In peripheral tissues, especially in skeletal muscle, APN activates AMPK, insulin receptor substrate-1 and fatty acid transport protein-1, to stimulate fatty acid combustion and glucose intake. It is interesting that these types of activation can be inhibited by tumor necrosis factor α (TNFα), another adipocytokine. Thus, it is assumed that APN deficiency affects the action elicited by other adipocytokines or the production of other adipocytokines, such as Pai-1. Therefore, we hypothesized that Pai-1 expression levels might also be depressed by APN.

In the present study, we aimed to clarify the bi-directional regulatory mechanisms between APN and Pai-1. We investigated the expression levels of APN and Pai-1 during 3T3-L1 pre-adipocyte differentiation, and examined the role of AMPK and PPARγ on APN and Pai-1 expression at the early and late differentiation stage. We demonstrated that APN can suppress Pai-1 expression through activation of AMPK, and Pai-1 can suppress APN expression through inhibition of a transcription factor, PPARγ.

Materials and Methods

Cell culture and induction of adipocyte maturation in the 3T3-L1 cell line. 3T3-L1 cells (JCRB Cell Bank, Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, UT, USA) (basal medium). Induction of differentiation into adipocyte phenotypes was performed by treating confluent cells with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich Co., St. Louis, MO, USA), 1 μM dexamethasone (Sigma-Aldrich) and 1.6 μM insulin (Life Technologies, Co., Carlsbad, CA, USA) in basal medium for two days (Figure 1A). After the treatment, the medium was replaced by basal medium, and the cells were incubated for three days (indicated as day 5 in Figure 1A) and 16 days (indicated as day 18 in Figure 1A).

Mouse recombinant adiponectin (R&D Systems Inc., Minneapolis, MN, USA), metformin (Wako Pure Chemical Industnes, Osaka, Japan), troglitazone (Sigma-Aldrich) and PNU74654 (Wnt-I; Sigma-Aldrich) were applied to adipocyte cells on day 2 and the cells were incubated until day 5. Mouse recombinant Pai-1 (Merck, Darmstadt, Germany) was applied to adipocyte cells on day 15 and the cells were incubated until day 18.

Mouse fat tissue samples. Five abdominal fat tissue samples from 15-week-old male APN-deficient mice and APN wild-type mice were obtained from our previous experiment reported elsewhere (10).

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] and Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific Inc., Waltham, MA, USA) added. Equal amounts of protein were separated in 5-20% gradient polyacrylamide gel electrophoresis-sodium dodecyl sulfate gels and transferred onto polyvinylidene difluoride membranes (Merck-Millipore, Billpore, MA, USA). Antibodies against p-AMPK and AMPK (Cell Signaling Technology, Danvers, MA, USA) were used at a 1:1,000 and 1:2,000 dilution, respectively. Blots were developed with enhanced chemiluminescence western blotting detection reagents (GE Healthcare, Buckingham Shire, UK).

Quantification of mRNA expression by quantitative real-time Polymerase Chain Reaction (qRT-PCR). Total RNA was isolated from cultured adipocyte and tissue samples using TRIzol Reagent (Invitrogen, Grand Island, NY, USA). One-microgram aliquots in a final volume of 20 μl were used for synthesis of cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was carried out using a CFX96™ (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with FastStart Universal SYBR Green MIX (×2) (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Primers for mouse *APN* (5' AGGATGCTA CTGTTGCAAGCTCTC, 5' CAGTCAGTTGG TATCATGGTAGAG), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; 5' TTGTCTC CTGCGACTTCA, 5' CACCACCCTGT TGCTGTA), Pai-1 (5' ACAGCCTTTGTCATCTCAGCC, 5' AGGG TTGCACTAAACAT GTCAG) were employed. The data were normalized by *GAPDH*. To assess the specificity of each primer set, amplicons generated from the PCR reaction were analyzed for melting curves.

Statistical analysis. Statistical analysis was performed using Student's *t*-test. Differences were considered to be statistically significant at $p<0.05$.

Results

Difference in APN and Pai-1 expression pattern during 3T3-L1 pre-adipocyte differentiation. 3T3-L1 is a suitable cell line to examine differences in molecular change during pre-adipocyte differentiation. Thus, expression levels of APN and Pai-1 were examined in 3T3-L1 cells at day 5 after the initiation of differentiation (early phase) and at day 18 after the initiation of differentiation (late phase). In the early phase, Pai-1 mRNA levels were significantly higher compared to those of undifferentiated 3T3-L1 cells (Figure 1B). In the late phase, *Pai-1* mRNA levels were significantly lower than those of undifferentiated 3T3-L1 cells. Comparing the expression levels of Pai-1 in early and late phases, an obvious reduction was observed in differentiated 3T3-L1 cells, while a slight induction of Pai-1 was observed in undifferentiated 3T3-L1 cells (Figure 1B). Comparing day 5 and day18, induction of APN was observed in both undifferentiated and differentiated 3T3-L1 cells in the late phase (Figure 1C). *APN* mRNA levels in differentiated cells tended to be lower compared to those of undifferentiated 3T3-L1 cells in the early phase, and higher when cells were in the late phase (Figure 1C and D).

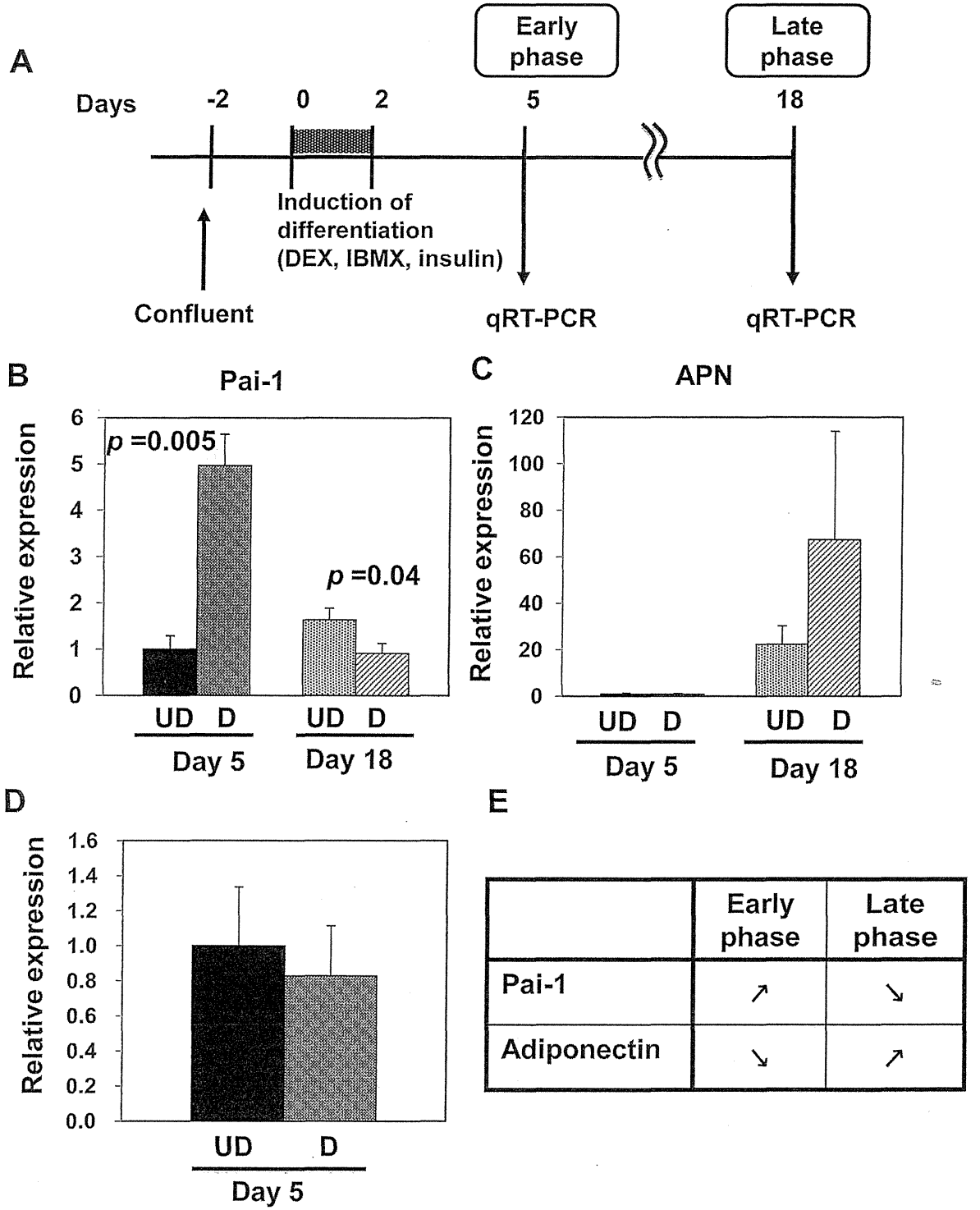

Figure 1. *Adiponecti n(APN) and plasminogen activator inhibitor-1(Pai-1) expression levels in 3T3-L1 pre-adipocytes. A: Illustration of differentiation protocol for 3T3-L1 pre-adipocytes is shown. Day 5 after the initiation of differentiation is defined as the 'early phase' and day 18 is defined as the 'late phase'. Quantitative real time-polymerase chain reaction (qRT-PCR) for Pai-1 (B) and APN (C) was performed at day 5 and day 18. The data are normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative Pai-1 and APN mRNA expression levels are plotted as the ratio of the untreated and undifferentiated control culture values. Data are means±SD (n=3). Similar results were obtained from more than three separate experiments. D: The data focused on low relative expression levels of Figure 1C. E: Summary of APN and Pai-1 expression patterns during 3T3-L1 pre-adipocyte differentiation. DEX, Dexamethasone; D, differentiated; IBMX, 3-isobutyl-1-methylxanthine; UD, undifferentiated.*

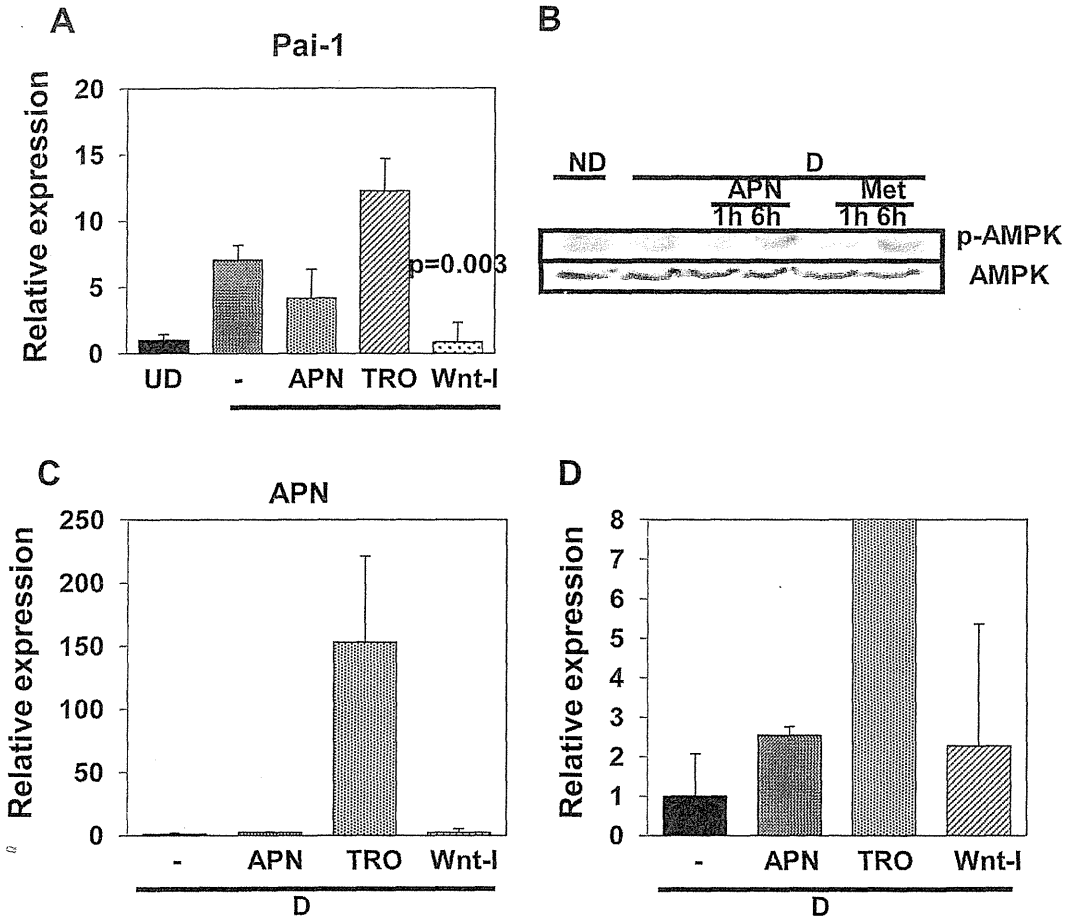

Figure 2. *Effects of adiponectin (APN), peroxisome proliferator-activated receptor (PPAR)γ ligand and Wnt inhibitor on 3T3-L1 cells at the early stage of differentiation. At day 2 after the initiation of differentiation, 3T3-L1 cells were treated with mouse recombinant protein APN (10 μg/ml), troglitazone (TRO), PPARγ ligand (10 μM) and Wnt inhibitor PNU74654 (20 μM) for three days. Quantitative real time-polymerase chain reaction (qRT-PCR) for Pai-1 (A) and APN (C and D) was performed. Relative Pai-1 and APN mRNA expression levels are plotted as the ratio of the untreated and undifferentiated control culture value. Data are means±SD (n=3). Similar results were obtained from three separate experiments. B: 3T3-L1 cells were treated with APN (10 μg/ml) and metformin (MET) an AMPK activator (5 mM) for 1 or 6 h, and AMPK and phosphorylated AMPK were examined by western blot. D: The data focused on low relative expression levels of Figure 1C. D, differentiated; UD, undifferentiated.*

Correlation between adiponectin and Pai-1 in the early phase. To clarify the relation between APN and Pai-1, 3T3-L1 cells were treated with APN at a dose of 10 μg/ml on day 2, after the initiation of differentiation. At the early-phase time point (day 5), high *Pai-1* mRNA expression levels were observed and it was found that APN could slightly reduce Pai-1 expression levels compared to those differentiated cells not treated with APN (Figure 2A). Phosphorylation of AMPK was confirmed by western blotting after six hours treatment with 10 μg/ml APN and 5 mM metformin, used as a positive control, at day 2 after the initiation of differentiation (Figure 2B). In addition, we tried to induce an increase in APN expression by treatment with troglitazone, a PPARγ ligand. As expected, treatment with 10 μM troglitazone markedly induced APN as shown in Figure 2C. However, treatment with troglitazone did not suppress but rather increased Pai-1 expression. On the other hand, we tried to reduce the high levels of Pai-1 by inhibiting Wnt/β-catenin signaling. PNU74654, a Wnt inhibitor, at a dose of 20 μM successfully suppressed Pai-1 expression levels (Figure 2A). APN expression levels under this treatment were examined, and almost a 2-fold elevation was observed (Figure 2C and D).

Correlation between adiponectin and Pai-1 in the late phase. To clarify the relation between APN and Pai-1 in the late phase, Pai-1 at a dose of 1 μg/ml was added to the medium at 15 days after the initiation of differentiation. At the late-phase time point (day 18), APN expression was slightly reduced by Pai-1 treatment compared to those of untreated

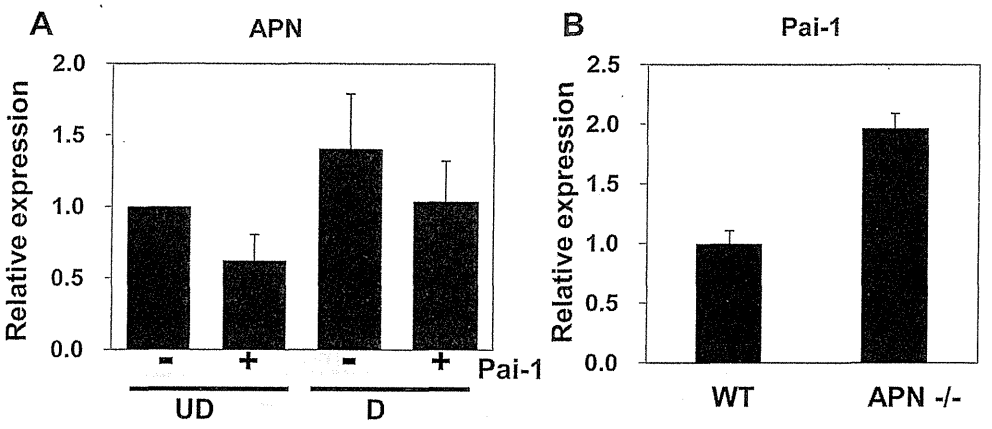

Figure 3. *Effects of plasminogen activator inhibitor-1 (Pai-1) on adiponectin (APN) mRNA levels and expression of Pai-1 in adiponectin-deficient mice. A: 3T3-L1 pre-adipocytes were treated with 1 μg/ml mouse recombinant Pai-1 at day 15 after the initiation of differentiation, and cells were collected for Quantitative real time-polymerase chain reaction (qRT-PCR) analysis at day 18. Relative APN mRNA expression levels are plotted as the ratio of the unstimulated-control culture value. Data are means±SD (n=3). Similar results were obtained from more than two separate experiments. B: qRT-PCR was performed on abdominal fat tissue from 12-week-old male adiponectin homozygous knockout mice (C57BL/6J background; n=5) and its control wild-type (WT) mice (n=5) as described in the Materials and Methods. Relative Pai-1 mRNA expression levels are plotted as the ratio of the value for the wild-type control fat tissue. Data are means±SD. APN(-/-), Homozygous adiponectin knockout mice; D, differentiated; UD, undifferentiated.*

differentiated and undifferentiated cells (Figure 3A). Furthermore, we confirmed an almost doubling of Pai-1 levels in the abdominal adipose tissue in *APN* homozygous knock-out mice compared to those of *APN* wild-type mice (Figure 3B).

Discussion

The present study demonstrated seesaw patterns of APN and Pai-1 expression in the different stages of pre-adipocyte differentiation. Moreover, bi-directional regulation observed between APN and Pai-1 may be through activation of AMPK and PPARγ (Figure 4).

In the early phase of 3T3-L1 cell differentiation, Pai-1 expression increased and APN slightly decreased. Besides, the late phase of differentiation showed low Pai-1 and high APN (Figure 1E). PPARγ, sterol regulatory element-binding protein-1c (SREBP-1c) and CCAAT/enhancer-binding proteins (C/EBP) are known to be involved in the early changes during pre-adipocyte differentiation (19). In the late phase of pre-adipocyte differentiation, the canonical Wnt signaling pathway reduces adipogenesis (19). These signalings might affect expression patterns observed for APN and Pai-1.

Indeed, a PPARγ ligand remarkably induced APN expression in this study, especially in the early phase (Figure 4A). Another PPARγ ligand, pioglitazone was also found to induce APN expression (20). Of note, Pai-1 is reported to suppress PPARγ expression (21). In this study, APN induction did not effectively lower Pai-1, but addition of 10 μg/ml APN to the culture medium did reduce Pai-1 expression. Concentrations of APN

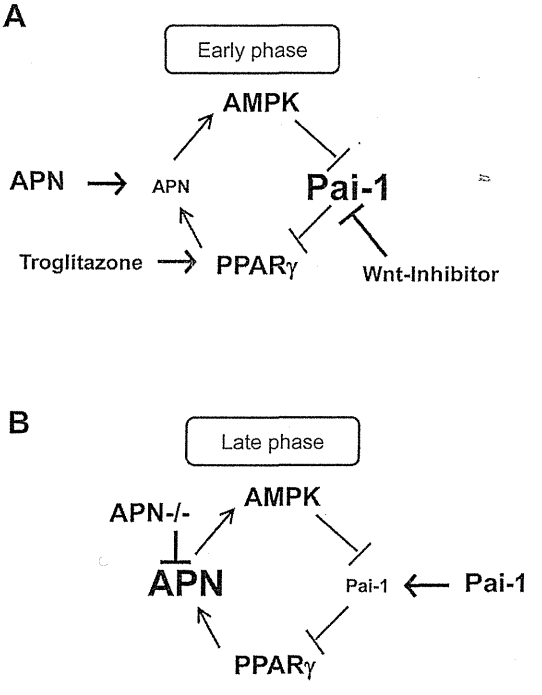

Figure 4. *Proposed mechanism for the seesaw regulation between adiponectin (APN) and plasminogen activator inhibitor-1 (Pai-1). A: Low APN and high Pai-1 expression levels in the early differentiation phase of 3T3-L1 cells may be regulated by AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor (PPAR)γ. Wnt inhibitor was used for suppression of Pai-1. Troglitazone was used for activation of PPARγ. APN was used for activation of AMPK. B: High APN and low Pai-1 expression levels in the late differentiation phase of 3T3-L1 cells may also be regulated by AMPK and PPARγ. Pai-1 was used for suppression of PPARγ. Abdominal fat tissue from APN-deficient mice (APN–/–) was used to determine the effects of APN deficiency on Pai-1 expression levels (→induction; —| suppression/inhibition).*

detected in plasma range from 3-30 μg/ml. Thus, a biologically appropriate dose might be used in this study. It has been reported that the activation of AMPK leads to the inhibition of adipogenesis (22). AMPK activation by APN resulted in suppression of Pai-1 expression. Similar findings were obtained in our recent experiment (10), in which APN-deficiency evoked hepatic Pai-1 induction. These findings suggest that in addition to the Pai-1-suppressive function of TNFα, APN-induced AMPK activation acts as a more direct physiological suppressor of Pai-1. Moreover, Wnt signal inhibitors lowered Pai-1 expression in the early phase. Pai-1 is reported to be a downstream target of Wnt/β-catenin signaling (17), and this might be the reason why APN is induced by a Wnt inhibitor. Summarizing effects in the early phase in 3T3-L1 cells, reduction of Pai-1 resulted in elevation of APN, and supplementation of APN resulted in reduction of Pai-1 (Figure 4A).

In the later phase of pre-adipocyte differentiation, low expression of Pai-1 and high expression of APN were observed. Addition of 1 μg/ml Pai-1 in the culture medium slightly reduced APN expression. Generally, the concentration of PAI-1 detected in human plasma is lower than 50 ng/ml. This dose may not be a biologically-appropriate dose, but may partly explain Pai-1 functions on specific occasions, such as in a localized area in the late phase of differentiation. We also examined the effect of APN-knockout conditions on Pai-1 expression using abdominal fat tissue samples from APN homozygous knock-out mice. Pai-1 expression was observed at a high level compared to that of fat tissue from APN wild-type mice. In the late phase of 3T3-L1 cells, supplementation of Pai-1 resulted in a slight reduction of APN (Figure 4B).

Both APN and Pai-1 are known to be involved in colorectal carcinogenesis. An absence of APN results in an increase of intestinal polyp development in *Min* mice (10), while a PAI-1 inhibitor was reported to reduce intestinal polyp development in *Min* mice (23). It is assumed that both induction of APN and inhibition/suppression of Pai-1 may be a useful approach in colorectal cancer prevention. Here, we have demonstrated a seesaw pattern of regulation between APN and Pai-1. Further studies are required to identify more direct regulatory mechanisms between APN and Pai-1, and the molecular targets identified might be utilized as novel chemopreventive targets.

Conflicts of Interest

None.

Acknowledgements

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Association of Pancreatic Fatty Infiltration With Pancreatic Ductal Adenocarcinoma

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OBJECTIVES: Fatty infiltration (FI) in the pancreas is positively correlated with high body mass index (BMI) or obesity, and the prevalence of diabetes mellitus (DM), which are well-known risk factors of pancreatic cancer. However, the association of FI in the pancreas with pancreatic cancer is unclear. Recently, we have shown that Syrian golden hamsters feature FI of the pancreas, the severity of which increases along with the progression of carcinogenesis induced by a chemical carcinogen. To translate the results to a clinical setting, we investigated whether FI in the pancreas is associated with pancreatic cancer in a series of patients who had undergone pancreatoduodenectomy.

METHODS: In the series, we identified 102 cases with pancreatic ductal adenocarcinoma (PDAC) and 85 controls with cancers except for PDAC. The degree of FI was evaluated histopathologically from the area occupied by adipocytes in pancreas sections, and was compared between the cases and controls.

RESULTS: The degree of FI in the pancreas was significantly higher in cases than in controls (median 26 vs. 15%, $P<0.001$) and positively associated with PDAC, even after adjustment for BMI, prevalence of DM and other confounding factors (odds ratio (OR), 6.1; $P<0.001$). BMI was identified as the most significantly associated factor with FI in the pancreas.

CONCLUSIONS: There is a positive correlation between FI in the pancreas and pancreatic cancer.

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INTRODUCTION

Pancreatic cancer is one of the most lethal human cancers with a 5-year survival rate of <5% in both Japan and the United States.[1] Thus, the development of useful predictive markers for individuals with a high risk of pancreatic cancer would be of great help in detecting pancreatic cancer at its early stages, and might contribute to a significant reduction of mortality. Epidemiological studies have shown that a family history of pancreatic cancer, cigarette smoking, age, obesity, and diseases such as chronic pancreatitis and diabetes mellitus (DM) increase the risk of pancreatic cancer.[2–4] A few pathologic studies of patients with pancreatic cancer have demonstrated fatty infiltraton (FI) in the pancreas parenchyma.[5,6] FI in the pancreas is positively correlated with age, body mass index (BMI), and a history of DM.[7–9]

Recently, we have shown that in Syrian golden hamsters, which exhibit a substantial age-related increase of hypertriglyceridemia and FI in the pancreas, there is further progression of pancreatic FI and carcinogenesis upon treatment with a carcinogen, *N*-nitrosobis (2-oxopropyl) amine (BOP), while the animals are fed a high-fat diet (HFD).[10] Therefore, we hypothesized that FI in the pancreas accompanied by hypertriglyceridemia might be associated with pancreatic cancer in both humans and experimental animals.

In the present case–control study, we examined whether FI in the pancreas is associated with pancreatic ductal adenocarcinoma (PDAC) in humans, independently of several other suggested risk factors for pancreatic cancer, such as obesity and DM.

METHODS

Patients and samples. Between January 2004 and December 2010, 367 patients underwent pancreatoduodenectomy for PDAC at the National Cancer Center Hospital, Japan. Among them, 102 were considered to be appropriate for the present study on the basis of the criteria detailed later. As controls, we used non-cancerous pancreas tissues from 85 patients who had undergone pancreatoduodenectomy for cancer, except for PDAC; these included 46 patients with distal bile duct cancer, 33 with cancer of the ampulla of Vater, 4 with gallbladder cancer, and 2 with duodenal cancer. DM was clinically diagnosed at the referring hospitals, using criteria of fasting blood glucose level ≥126 mg/dl and HbA1c ≥6.1%, before the patients visited our hospital to resect pancreatic cancer. BMI was calculated when the patients were admitted to our hospital. The use of each individual's material for analysis in the

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present study was approved by the Ethics Review Committee of the National Cancer Center (2010-088). The materials are from patients who had given general consent for the research use of their leftover samples, and all clinical investigations were conducted in accordance with the principles of the Declaration of Helsinki.

Pathological examination. PDACs were examined pathologically and classified according to the World Health Organization classification and TNM classification.[11,12] Surgically resected specimens were fixed in 10% formalin, and the pancreas heads were cut horizontally into serial slices 5 mm thick. In order to evaluate FI appropriately, we conducted a preliminary study to select a target area of pancreas parenchyma in 16 cases of PDAC. As FI is easily affected by any type of pancreatitis associated with cancer infiltration, including obstructive pancreatitis, we selected the FI area for measurement, avoiding any primary and/or secondary effect caused by cancer infiltration (Supplementary Figure S1 online). Thus, pancreatitis patients were ruled out from the FI evaluation. First, we selected anterior and cranial areas of the pancreas near the duodenum that correspond to the dorsal pancreas during organogenesis. Second, we chose areas of the pancreas near the ampulla of Vater if the former area was affected by cancer infiltration. If both of these areas were affected by cancer infiltration, such cases were excluded from the study. Thus, we selected one section containing non-tumorous pancreatic tissue and confirmed whether it fulfilled the above conditions. Then, FI areas were measured quantitatively as the percentage of area infiltrated by adipocytes relative to the total area on the section was calculated using the WinROOF image analysis software package (Mitani Corp, Tokyo, Japan). The reproducibility of this quantitation method was checked preliminarily by comparing the FI area of one section with another section derived from tissue immediately adjacent to the former. The difference between the two FI area values measured in 16 pairs of sections was 5.6% on average.

Serum sample collection and assays. Peripheral blood was collected from each patient at the time of the hospital visit prior to treatment, and blood sugar, HbA1c, and serum levels of total cholesterol (TC), high-density lipoprotein (HDL), amylase, CEA, and CA19-9 were measured by participants at the National Cancer Center Hospital. For further examination, serum provided by the National Cancer Center Biobank, Japan, was stored at −20°C. Serum adiponectin, leptin and insulin growth factor-I (IGF-I) (R&D Systems, Inc., Minneapolis, MN, USA), apolipoprotein A-II (apoA-II) (Assay pro, St Charles, MO, USA), insulin (Millipore, Billerica, MA, USA), and serum amyloid A (SAA; Invitrogen, Camarillo, CA, USA) were measured using enzyme-linked immunosorbent assay kits in accordance with the manufacturers' instructions. The levels of serum triglycerides (TGs), HDL, and gamma-glutamyltransferase (GGT) were analyzed using the FUJI Dri-Chem system (Fuji Film, Tokyo, Japan).

Statistical analysis. The cases and controls were classified into three subgroups, <10%, 10–20%, and ≥20%, according to the area of FI. The cutoff points of 10 and 20 were nearly equal to the tertile cutoff points in the controls, namely 9.5 and 20.4. An unconditional logistic regression model was used to estimate odds ratios (ORs) and their 95% confidence intervals (CIs) of PDAC according to the three categories of FI in the pancreas, the lowest value being used as a reference. Two-sided P values <0.05 were considered to indicate statistical significance. All statistical analyses were carried out using the Statistical Analysis System (SAS), version 9.1 software package (SAS Institute, Cary, NC, USA) by a statistician (T.Y.).

RESULTS

Patient characteristics. Among 102 cases, one was classified as stage IB, 27 as stage IIA, 65 as stage IIB, and 9 as stage IV. The characteristics of the case and control patients are summarized in Table 1. Controls were older than cases ($P=0.001$), and there was a male predominance in both groups. The known risk factors for PDAC were compared between cases and controls. The prevalence of DM ($P=0.03$) and family history of pancreatic cancer ($P=0.007$) in cases was higher than in controls. The values of blood sugar ($P=0.002$) and HbA1c ($P<0.001$) in cases were also significantly higher than in the controls. The serum apoA-II level was shown to be lower in cases than in controls ($P=0.02$), as reported previously, in comparison with healthy subjects. CEA ($P=0.04$) and CA19-9 ($P<0.001$), serum tumor markers for PDAC, were also significantly higher in cases than in controls. Meanwhile, serum levels of GGT ($P<0.001$), which are associated with liver and biliary disorders, were higher in controls than in cases.

Association of FI in the pancreas with PDAC. In the human pancreas, adipocytes were observed to accumulate in the area between pancreatic lobules (interlobular fat), especially around great vessels, or to be scattered in the lobules (intralobular fat), as shown in Figure 1. The distribution pattern of FI in some patients was similar to that observed in hamster pancreas.[10] In this study, FI in the pancreas was defined as the sum of the areas showing any types of FI in the pancreas parenchyma. Table 1 shows that the area of FI in the pancreas was significantly greater in cases than in controls (median 26 vs. 15%, $P<0.001$). Types of differentiation and stages of PDACs were not associated with the degree of FI (data not shown).

Table 2 shows the association between the area of FI in the pancreas and PDAC. A significantly higher OR for PDAC was observed according to the area of FI in the pancreas ($P<0.001$). Adjusted for sex, age, BMI, history of DM, and family history of pancreatic cancer, confounding factors for pancreatic cancer, ORs for PDAC showed an increasing trend according to the area of FI ($P<0.001$). Even when patients with a BMI >25 kg/m^2, a history of DM, and a family history of pancreatic cancer were excluded, positive associations between the degree of FI in the pancreas and PDAC were observed ($P<0.001$ overall).

The factors associated with FI. The characteristics of the study participants were examined in relation to the degree of FI of the pancreas in controls and cases and are shown in Supplementary Tables 1 and 2, respectively. BMI and age were positively correlated with the area of FI of the pancreas

Table 1 Selected characteristics of study subjects

Characteristic	Cases ($n=102$)	Controls ($n=85$)	P^a
Categorical variables, n *(%)*			
FI in the pancreas ≥ 20%	64 (62.7)	30 (35.2)	<0.001
Male	60 (58.8)	60 (70.5)	0.12
Ever smoking	53 (51.9)	42 (49.4)	0.77
Frequent drinking (5–7 times/week)	34 (33.3)	31 (36.9)	0.85
DM	30 (29.4)	14 (16.4)	0.03
Hypertension	36 (35.2)	27 (31.7)	0.64
Hyperlipidemia	7 (6.8)	10 (11.7)	0.30
Family history of PC	11 (10.7)	1 (1.1)	0.007
Continuous variables, median (IQR)			
FI in the pancreas, %	25.8 (14.2–40.9)	15.0 (7.7–24.8)	<0.001
Age, years	63.5 (56–69)	68.0 (63–73)	0.001
BMI, kg/m^2	22.4 (20.3–24.3)	22.7 (20.7–24.2)	0.95
Blood sugar, mg/dl	114.0 (100–141)	106.0 (93–119)	0.002
HbA1c, %	5.5 (5.1–6.4)	5.1 (4.7–5.5)	<0.001
TC, mg/dl	189.0 (162–221)	195.0 (169–227)	0.31
HDL, mg/dl	52.0 (43–62)	52.0 (42–67)	0.47
TG, mg/dl	149.0 (109–209)	155.0 (117–210)	0.38
Apo A-II, μg/ml	219.3 (136.7–397.5)	327.3 (174.0–444.4)	0.02
Adiponectin, μg/ml	5.4 (3.0–9.6)	6.3 (3.2–12.3)	0.37
Leptin, ng/ml	3.2 (2.2–4.6)	3.1 (2.4–3.8)	0.57
Insulin, mU/l	3.5 (2.5–5.8)	3.7 (2.8–6.4)	0.41
IGF-I, ng/ml	69.7 (53.2–93.5)	74.1 (54.4–96.4)	0.69
Amylase, IU/l	107.0 (75–182)	105.0 (83–141)	0.55
CEA, ng/ml	2.6 (1.6–4.1)	2.0 (1.3–3.4)	0.04
CA19-9, U/ml	96.0 (46–400)	30.0 (16–121)	<0.001
SAA, μg/ml	22.1 (8.93–54.3)	35.8 (12.7–89.8)	0.06
GGT, ng/ml	105.0 (33–311)	339.0 (101–673)	<0.001

Apo A-II, apolipoprotein A-II; BMI, body mass index; DM, diabetes mellitus; FI, fatty infiltration; GGT, gamma-glutamyltransferase; HDL, high density lipoprotein; IGF-I, insulin growth factor-I; IQR, interquartile range; PC, pancreatic cancer; SAA, serum amyloid A; TC, total cholesterol; TG, triglyceride.
[a]Based on the Fisher's exact test for percentage difference and the Wilcoxon rank-sum test for median difference.

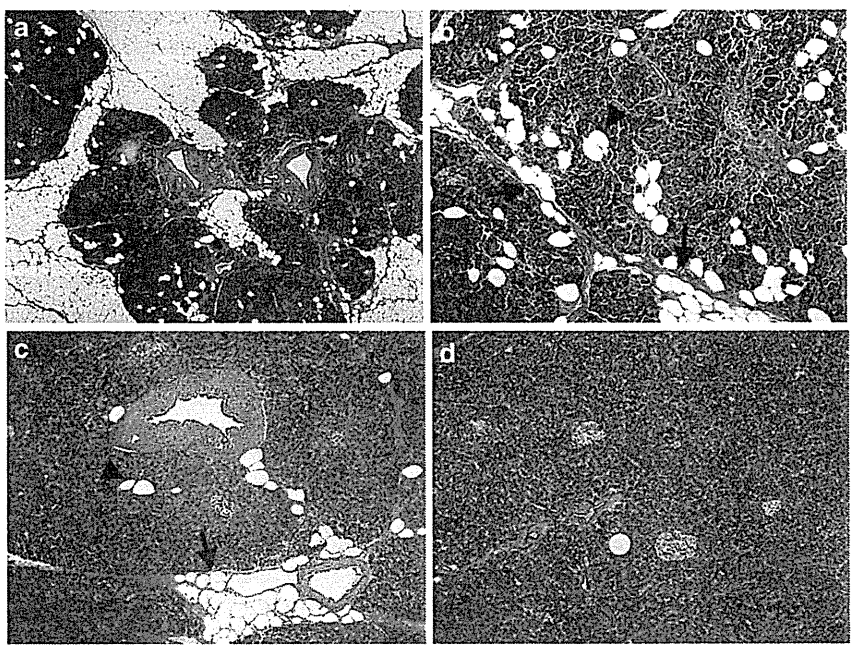

Figure 1 Histology of the human pancreas with fatty infiltration. (**a**, **b**) Pancreas tissue with moderate to severe FI. Most of the pancreas parenchyma has been replaced by adipocytes, and the remaining pancreas lobules resemble islets surrounded by a fatty lake. Most adipocytes have accumulated interlobularly (arrow in **b**), but some are scattered within the lobules (arrowhead in **b**). (**c**) Pancreas tissue with mild FI. Adipocytes have accumulated around arterioles (arrow), and several adipocytes are scattered within the lobules (arrowhead). (**d**) Pancreas tissue with minimal FI. Super-low magnification in **a**, and low magnification in (**b** to **d**).

Table 2 Association of the degree of FI in the pancreas with pancreatic ductal adenocarcinoma

	FI in the pancreas						
	<10%		≥10%, <20%		≥20%		
Population	**OR**	**95% CI**	**OR**	**95% CI**	**OR**	**95% CI**	***P*[a]**
All subjects							
Cases/controls		17/30		21/25		64/30	
Crude estimate	1	Reference	1.4	(0.6–3.4)	3.7	(1.8–7.8)	<0.001
Adjusted estimate[b]	1	Reference	2.3	(0.8–6.2)	6.1	(2.4–15.2)	<0.001
Excluding those with BMI of ≥25 kg/m²							
Cases/controls		17/28		18/22		49/24	
Adjusted estimate[c]	1	Reference	2.1	(0.7–5.9)	6.3	(2.4–16.5)	<0.001
Excluding those with past history of DM							
Cases/controls		14/28		18/21		40/22	
Adjusted estimate[d]	1	Reference	3.1	(1.0–9.3)	7.5	(2.6–21.3)	<0.001
Excluding those with family history of PC							
Cases/controls		15/29		17/25		59/30	
Adjusted estimate[e]	1	Reference	2.0	(0.7–5.5)	5.4	(2.2–13.6)	<0.001

BMI, body mass index; CI, confidence interval; DM, diabetes mellitus; FI, fatty infiltration; OR, odds ratio; PC, pancreatic cancer.
[a]Statistical tests for trend (two-sided) were assessed by assigning ordinal values to the degree of FI in the pancreas.
[b]Adjusted for sex, age (≤60, 61–70 and >70), BMI (<25, ≥25), past history of DM (yes or no) and family history of PC (yes or no).
[c]Adjusted for sex, age (≤60, 61–70 and >70), past history of DM (yes or no), and family history of PC (yes or no).
[d]Adjusted for sex, age (≤60, 61–70 and >70), BMI (<25, ≥25), and family history of PC (yes or no).
[e]Adjusted for sex, age (≤60, 61–70 and >70), BMI (<25, ≥25), and past history of DM (yes or no).

in both cases and controls. In control patients, the serum TG and amylase values were also positively correlated with the area of FI in the pancreas. Meanwhile, the levels of the serum insulin, HbA1c and blood sugar in case patients were positively correlated with the area of FI in the pancreas. To further investigate an association with FI in the pancreas, we conducted a multivariable linear regression analysis in each group, in which the above variables (BMI, serum TG, and amylase for controls; BMI, serum insulin, HbA1c, and blood sugar for cases), as well as age and sex, were included in one model. After mutual adjustment, a statistically significant association was noted only for BMI (controls, $P=0.001$; cases, $P=0.01$).

DISCUSSION

Based on epidemiological observation of human pancreatic cancers, FI in the pancreas was suggested to associate with PDAC, independently of known risk factors such as obesity and DM (Supplementary Figure S2). Although we identified BMI, a measurement of obesity, as the most significantly associated factor among several factors related to FI in the pancreas, FI in the pancreas was likely to increase the risk of pancreatic cancer beyond the effect of obesity alone. Some previous studies have evaluated pancreatic FI in humans using diagnostic modalities such as ultrasound, magnetic resonance imaging, or magnetic resonance spectroscopy.[9,13–16] FI in the pancreas has been suggested to promote dissemination and lethality of PDAC and to increase the risk of postoperative pancreatic fistula.[17,18] Here we demonstrated that the area of FI in histopathological sections of PDAC resected can be used as a quantitative indicator of the degree of FI. This is the first report to indicate an association between the area of FI and the development of PDAC.

Although mechanistic insights into how PDAC could develop from such an adipocyte-rich microenvironment are not clear, recent evidence suggests that ectopic fat accumulation produces certain adipocytokines that induce cell proliferation.[19,20] Serum adipocytokine levels were not clearly correlated with the area of FI in the present study, but the level of leptin expression was high in the pancreas of BOP-treated hamsters fed a HFD.[10] Thus, local release of adipocytokines from adipocytes in an adipocyte-rich microenvironment appeared to be correlated with PDAC development.

In the present study, serum insulin levels in cases were positively correlated with FI in the pancreas. It has also been reported that HOMA-IR is strongly correlated with FI of the pancreas except in subjects with a history of DM, pancreatic diseases and liver diseases.[9] In an *in vitro* setting, it has been shown that glucose-dependent insulinotropic polypeptide activates lipoprotein lipase, leading to TG accumulation in differentiated 3T3-L1 adipocytes in the presence of insulin.[21] Therefore, it is conceivable that induction of high glucose and insulin levels by hyperphagia could be associated with FI through activation of lipoprotein lipase in the pancreas. Conversely, it has also been suggested that increased pancreatic FI is related to β-cell dysfunction in the absence of type 2 DM,[22] and that this can lead to subsequent development of type 2 DM.[23,24] The hyperinsulinemia seen in human obesity, including the early phase of type 2 DM, may be closely related to FI in the pancreas.

Several possible mechanisms underlying the development of FI in the pancreas can be speculated. It has been shown in experimental animal models that FI can be induced in the pancreas by obstruction of the pancreatic duct or vasculature.[25,26] Smits and van Geenen[27] have showed that FI or non-alcoholic fatty pancreas disease represents fat accumulation induced by obesity and metabolic syndrome, while fatty replacement represents replacement of adipocytes induced by

death of acinar cells. We agree with their statements that pancreatic fat accumulation is mainly induced by these two factors. In this study, pancreatic FI in cases represents any type of fat accumulation caused by any type of etiology. It has been reported that lipotoxicity caused by a high TG content induces inflammatory responses and necrosis in pancreatic acinar cells *in vitro*.[28,29] It has also been shown that c-Myc activity is required for growth and maturation of the exocrine pancreas and for the transdifferentiation of acinar cells into adipocytes in mice.[30] Thus, pancreas containing scattered adipocytes might be more sensitive to acinar cell damage due to lipotoxity and other genetic factors, and scattered FI may reflect the acinar cell death or transdifferentiation after the damage.

Some limitations could be pointed out in this study. The major limitation is that it lacked normal healthy controls because pancreatic sections could be obtained only from patients who had undergone pancreatoduodenectomy. A second limitation is that we could not measure FI in more than one pancreatic section, as areas for measuring FI were limited and small because the areas of tumor and secondary inflammation were avoided. Therefore, a future study using a non-invasive method will be required to evaluate FI in a large area/volume of pancreas from healthy and case subjects. Previously, we have reported a case of PDAC that was associated with marked FI in the pancreas, as seen on computed tomography images.[31] Computed tomography imaging of the pancreas would be a useful approach for accurate evaluation and follow-up of pancreatic FI in normal subjects, as well as in cohort studies. The third limitation is that we did not exclude the areas of pancreas with PanINs from the sections for measuring FI because it is known that PanINs are sometimes found in pancreatic tissue of the elderly, and also that a large number of PanINs with various grades are found in the pancreas of the patients with PDAC. Therefore, it is extremely difficult to measure FI in the pancreas tissue without PanINs, especially in the limited area for measuring FI. The fourth limitation is that BMI could be underestimated in the cases, because weight loss is a very common symptom of patients suffering from pancreatic cancer even though most cases were classified as stage IIA or IIB. The fifth limitation is that there is no validation study. To confirm the observation in the present study, the same study should be repeated with the same methods in another center (hospital/institution). The final limitation is that we cannot distinguish whether FI was a risk factor or a consequence of the cancer. The only way to demonstrate that FI is a risk factor for PDAC is to perform a prospective cohort study to observe whether individuals with fatty pancreas could develop PDAC. For this purpose, we are now trying to establish the methods to evaluate FI in a large area/volume of pancreas by non-invasive method, using computed tomography and magnetic resonance imaging. In addition, studies on pancreatic carcinogenesis using animal models of fatty pancreas would be helpful to elucidate underlying mechanisms.

In conclusion, there is a positive correlation between FI in the pancreas and pancreatic cancer. The development of effective detection methods and/or markers of FI, especially "fatty pancreas" with severe FI, is warranted for mass screening of individuals at high risk of pancreatic cancer at health examinations.

CONFLICT OF INTEREST

Guarantor of the article: Hitoshi Nakagama, MD, DMSc.

Specific author contributions: Mika Hori contributed to the design of the study, acquisition, analysis and interpretation of data, writing and drafting of the manuscript; Mami Takahashi contributed to the conception of the study, development of methodology and data analysis and revision of the manuscript; Nobuyoshi Hiraoka contributed to the histopathological analysis and revision of the manuscript; Taiki Yamaji contributed to the statistical analysis and revision of the manuscript; Michihiro Mutoh contributed to data analysis and revision of the manuscript; Rikako Ishigamori contributed to the histopathological analysis; Koh Furuta contributed to material supports in human serum analysis; Takuji Okusaka contributed to the clinical revision of the manuscript; Kazuaki Shimada contributed to the clinical revision of the manuscript; Tomoo Kosuge contributed to the clinical revision of the manuscript; Yae Kanai contributed to the histopathological analysis; Hitoshi Nakagama contributed to study supervision and revision of the manuscript.

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Potential competing interests: None.

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Study Highlights

WHAT IS CURRENT KNOWLEDGE

- Fatty infiltration (FI) in the pancreas is positively correlated with obesity and prevalence of DM.
- The association of FI in the pancreas with pancreatic ductal adenocarcinoma (PDAC) is unclear in humans.

WHAT IS NEW HERE

- FI in the pancreas is associated with PDAC development in humans.
- Body masss index (BMI) was identified as the most significantly associated factor with FI in the pancreas.
- FI in the pancreas may increase the risk of PDAC beyond the effect of obesity alone.

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