

High susceptibility to azoxymethane-induced colorectal carcinogenesis in obese KK-*A^y* mice

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Obesity is associated with colon carcinogenesis. However, not much information is available regarding the mechanisms of obesity-associated colorectal cancer, and there are only few useful animal models for investigating the underlying mechanism between obesity and colorectal cancer. KK-*A^y* mice exhibit severe obesity. Amount of visceral fat assessed by micro-computed tomography was almost 15 times higher than that of same aged C57BL/6J mice. Treatment with azoxymethane (AOM; 200 µg/mouse injected once a week for 3 times) resulted in markedly increased colon aberrant crypt foci (ACF) development (≈70 ACF/mouse) in KK-*A^y* mice compared with lean C57BL/6J mice (≈9 ACF/mouse). Moreover, administration of AOM at a dose of 200 µg/mouse once a week for 6 times developed colorectal adenocarcinomas within only 7 weeks after the last AOM injection. The incidence of adenocarcinoma was 88% in KK-*A^y* mice and was markedly higher than the 4% observed in C57BL/6J mice. The number of tumors/mouse was 7.80 in KK-*A^y* mice and also markedly higher than the 0.12 in the C57BL/6J case. Interestingly, adenocarcinomas were observed in most of the AOM-treated KK-*A^y* mice along with remarkable tumor angiogenesis, and some showed submucosal invasion. These results indicate that the KK-*A^y* mouse, featuring intact leptin and leptin receptor Ob-Rb1, could be a useful animal model to investigate obesity-associated cancer.

Epidemiological studies have suggested that metabolic syndrome, characterized by hyperglycemia, hyperinsulinemia, hyperlipidemia and hypertension, is a risk factor for colorectal cancer.^{1,2} Patients with Type 2 diabetes mellitus also have a higher risk of colon cancer.¹ In spite of a number of epidemiological studies accumulated, the mechanisms of obesity-associated colorectal cancer have not been fully

understood. Moreover, there are only few useful animal models for investigating the underlying mechanism between obesity and colorectal cancer. Thus, it is very important to establish a useful animal model for this purpose.

Key words: obesity, colorectal carcinogenesis, adipocytokine

Abbreviations: ACF: aberrant crypt foci; AOM: azoxymethane; CYP2E1: cytochrome P450 2E1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; IL-6: interleukine-6; MCP-1: monocyte chemoattractant protein-1; Pai-1: plasminogen activator inhibitor-1; TNF-α: tumor necrosis factor-α; VEGF: vascular endothelial growth factor

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Grants-in-Aid for Cancer Research, Ministry of Health, Labour, and Welfare of Japan (Third-Term Comprehensive 10-Year Strategy for Cancer Control), Grants-in-Aid for Foundation for Promotion of Cancer Research

DOI: 10.1002/ijc.25711

History: Received 19 May 2010; Accepted 16 Sep 2010; Online 30 Sep 2010

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Obesity animal models including *ob/ob* mice, *db/db* mice and Zucker rats are well established,³⁻⁵ and it has been reported that intraperitoneal injection of the colorectal-specific carcinogen azoxymethane (AOM) to *ob/ob* and *db/db* mice resulted in the development of around 15 colorectal aberrant crypt foci (ACF).⁶ Carcinogen-induced colon carcinogenesis has also been described in Zucker rats.⁷ However, these animals lack leptin or have mutation that inactivates Ob-Rb1, the long form of the leptin receptor with signaling potential.³⁻⁵ On the other hand, short Ob-R isoforms lack major domains recruiting downstream effectors and have diminished or abolished signaling potential.³ Evidence is accumulating that leptin can promote colorectal cancer development through activation of the NF-kappaB, Erk1/2 and PI3K/Akt pathways.⁸ Therefore, it is desirable to use animals with intact leptin and leptin receptor Ob-Rb1 to investigate obesity-associated colorectal cancer.

The KK-*A^y* mice were established by cross-mating KK mice, Type 2 diabetes model mice, with C57BL/6J-*A^y* mice,⁹ which carry the Agouti gene (*A^y*), to induce severe hyperphagia, polydipsia, impaired glucose tolerance, hyperinsulinemia and hyperlipidemia¹⁰ as compared with C57BL/6J mice, which are generally used as non-obese, non-diabetic controls.¹¹⁻¹³ Moreover, KK mice feature intact leptin and leptin receptor.¹⁴

In this study, we aimed to investigate the effects of the obesity on AOM-induced colorectal ACF and cancer development in KK-*A*^y mice. Great susceptibility to AOM-induced colorectal ACF and cancer demonstrated that the KK-*A*^y mouse is a good model for human metabolic syndrome. The utility of KK-*A*^y mice for investigation of mechanisms of how obesity enhances colorectal carcinogenesis is also discussed with reference to adipocytokine production.

Material and Methods

Animals

Female 5-week-old KK-*A*^y/TaJcl (KK-*A*^y) mice and C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan), and acclimated to laboratory conditions for 1 week. Five mice were housed per plastic cage with sterilized softwood chips as bedding in a barrier-sustained animal room at 24°C ± 2°C and 55% humidity on a 12 hr light/dark cycle and fed AIN-76A powdered basal diet (CLEA Japan). Food and water were available *ad libitum*. The animals were observed daily for clinical signs including anal bleeding and mortality. Body weights and food and water consumption were measured weekly. 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.

AOM-induced colorectal aberrant crypt focus development

For the induction of ACF by AOM (Nard Institute, Amagasaki, Japan), 6-week-old female KK-*A*^y (*n* = 10) and C57BL/6J (*n* = 10) mice were given intraperitoneal injections of AOM (200 µg/mouse) once weekly for 3 weeks. Five mice each were also injected with saline as a control group. At the end of the experimental period, the colorectum was removed, opened longitudinally and fixed flat between sheets of filter paper in 10% buffered formalin for more than 24 hr. They were divided into the proximal segment, rectum (1.5 cm in length), then the proximal (middle) and distal halves of the remainder. These were stained with 0.2% methylene blue (Merck, Darmstadt, Germany) and the mucosal surface was assessed for ACF with a stereoscopic microscope, as previously reported.¹⁵

AOM-induced colorectal tumor development

Six-week-old female KK-*A*^y (*n* = 25) and C57BL/6J (*n* = 25) mice were given intraperitoneal injections of AOM (200 µg/mouse) once weekly for 6 weeks for induction of colorectal tumors. Five mice each were also injected with saline as a control group. All the mice were anesthetized with ether and sacrificed at the age of 19 weeks, because bloody stool then became frequently observed. The colorectum was opened longitudinally and colorectal tumors were noted for their location, number and size. Colorectal tumors along with nontumorous parts were fixed in 10% buffered formalin and embedded in paraffin blocks for histopathological evaluation.

Diagnosis of colorectal tumors using hematoxylin and eosin (H&E) stained sections was performed according to the classification of Pozharisski.¹⁶ The organs, including heart, kidney, liver, lung, pancreas and spleen, were also excised and were also observed macroscopically and blood samples from the abdominal aorta were collected. Abnormal findings were further histopathologically examined. Serum levels of triglyceride, total cholesterol and lipoproteins were measured as reported.¹⁷ Blood glucose was measured using a GR-102 blood glucose monitor (Terumo, Tokyo, Japan).

Analysis of visceral adiposity

The volume and distribution of visceral fat were measured by LaTheta[®] X-ray computed tomography (CT; Aloka, Tokyo, Japan) from the first lumbar vertebra to the pubic bone, under inhalation anesthesia of isoflurane and were analyzed by visualization LaTheta[®] software as previously reported.¹⁸

Analysis of mRNA levels and serum adipocytokine levels

Visceral adipose tissue and colon mucosa of KK-*A*^y and C57BL/6J mice were rapidly deep-frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated from tissue by using an RNeasy[®] Lipid Tissue mini kit (Qiagen, Hilden, Germany) according to the manufacture's protocol, and treated with DNase I (Invitrogen, Carlsbad, CA). One-µg RNA in a final volume of 20 µL were used for synthesis of cDNA using an Omniscript[®] RT Kit (Qiagen, Hilden, Germany) with an oligo (dT) primer. Real-time PCR was carried out using a DYA Engine Opticon[™] 2 (MJ Japan, Tokyo, Japan) with SYBR Green Real-time PCR Master Mix (Toyobo Co., Osaka, Japan). Primers for mouse adiponectin (5' primer- AGGATGCTACTG TTGCAAGCTCTC, 3' primer- CAGTCAGTTGGTATCATGG TAGAG), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5' primer- TTGTCTCCTGCGACTTCA, 3' primer- CACCAC CCTGTTGCTGTA), interleukine-6 (IL-6) (5' primer- ACAAC CACGGCCTTCCCTACTT, 3' primer- CACGATTTCCAGAG AACATGTG), leptin (5' primer- CCAAACCCCTCATCAA GACC, 3' primer- GTCCAAGTGTGAAGAATGTCCC), monocyte chemoattractant protein-1 (MCP-1) (5' primer- CCACTCACCTGCTGCTACTCAT, 3' primer- TGGTGATC CTCTTGTAGCTCTCC), Ob-Rb1 (5' primer- CCATCTTTA TATGATCTGCCTGAAGT, 3' primer- TGCATTGGACAGT CTGAAAGCT), plasminogen activator inhibitor-1 (Pai-1) (5' primer- ACAGCCTTTGTCATCTCAGCC, 3' primer- AGGGTTGCACTAAACATGTCAG) and tumor necrosis factor-α (TNF-α) (5' primer- TGTGCTCAGAGCTTTCAACAAC, 3' primer- GCCCATTTGAGTCCTTGATG) were used.¹⁹⁻²² To assess the specificity of each primer set, amplicons generated from the PCR reaction were analyzed for melting curves.

Serum insulin, IL-6, leptin, Pai-1, resistin and TNF-α were measured using Multiplex kits (Linco plex, St. Louis, MO).

Immunohistochemical analysis of infiltrated macrophages in visceral fat tissues

Visceral fat tissues, represented by peri-uterine and peri-ovarian white adipose tissues from KK-*A*^y and C57BL/6J mice were

fixed in 10% buffered formalin, embedded, and sectioned for further immunohistochemical examination with the avidin-biotin complex immunoperoxidase technique. As the first antibody, goat polyclonal anti-F4/80 antibody (R&D Systems, Minneapolis, MN) was used at 100 \times dilution. As the secondary antibody, biotinylated anti-goat IgG (Vector Laboratories, Burlingame, CA) was used at 200 \times dilution. Staining was performed using avidin-biotin reagents (Vectastain ABC reagents; Vector Laboratories, Burlingame, CA), 3,3'-diaminobenzidine and hydrogen peroxide, and the sections were counterstained with hematoxylin to facilitate orientation. Three different areas of tissue from each animal ($n = 5$) were photographed in 20 \times magnification, and the numbers of F4/80 positive cells were counted manually.

Analysis of crypt length and cells/crypt

The crypt length and the number of cells/crypt were evaluated on H&E stained sections from similar distal parts of nontumorous mucosa of 19 weeks KK-A^y and C57BL/6J mice with AOM treatment. Three different crypts from each animal ($n = 5$) were photographed in 40 \times magnification, and the crypt length were measured and the number of cells/crypt were counted.

Analysis of hepatic CYP2E1 activity

Liver microsomes were prepared from liver samples from 19-week-old KK-A^y and C57BL/6J mice with or without AOM treatment ($n = 5$ each), and cytochrome P450 2E1 (CYP2E1) activity of liver microsomes was measured using the method described by Bonnefoi *et al.*²³ with some modifications. CYP2E1 activity was expressed as the formation rate of 6-hydroxychlorzoxazone from chlorzoxazone. Mixture of 100 μ g microsomes, 20 μ L of 500 mM phosphate buffer (pH 7.4) and 20 μ L of 500 μ M chlorzoxazone solution were preincubated for 5 min at 37°C (total volume: 180 μ L). Then, 20 μ L of NADPH-generating system (100 mM MgCl₂, 10 mM NADP, 100 mM G-6-P, and 1000 U/mL G-6-PDH) were added and incubated for 5 min at 37°C. After incubation, the reaction was stopped by adding 100 μ L of ice-cold acetonitrile. The concentration of formed 6-hydroxychlorzoxazone in each solution was determined with HPLC equipped with a model LC-6A pump (Shimadzu Co., Kyoto, Japan) and a model SPD-6AV UV detector (Shimadzu Co.). A UV wavelength of 295 nm was used. Each sample was chromatographed on a CAPCELL PAK C-18-UG120 (4.6 mm i.d., 150 mm in length, Shiseido Co., Tokyo, Japan). The flow rate was 1.0 mL/min. The mobile phase was a mixture of acetonitrile and 20 mM acetate buffer (pH 4.5) (30/70, v/v).

Statistical analysis

The results are expressed as mean \pm standard deviation (SD) or standard error (SE) values. The significance of difference in the incidence of AOM-induced mouse tumors was analyzed using the χ^2 test and other statistical analyses were performed with Student's *t*-test. Differences were considered to be statistically significant at $p < 0.05$.

Results

Obese status observed in KK-A^y mice

The average body weights at 6 weeks of age of female KK-A^y and C57BL/6J mice were 22.9 \pm 0.9 g (mean \pm SD) and 17.4 \pm 0.8 g, respectively, before AOM treatment. At 13 weeks of age (ACF experiment), the average body weights of KK-A^y and C57BL/6J mice with AOM treatment were 40.6 \pm 2.7 g (mean \pm SD) and 21.4 \pm 1.5 g, and food intake were 4.0 \pm 0.5 g/mouse/day and 3.0 \pm 0.2 g/mouse/day, respectively. At 19 weeks of age (colorectal cancer experiment), the average body weights of KK-A^y and C57BL/6J mice with AOM treatment were 55.4 \pm 4.9 g and 25.2 \pm 1.5 g, and food intake were 5.2 \pm 0.5 g/mouse/day and 3.6 \pm 0.4 g/mouse/day, respectively. The average body weights of mice treated with AOM were slightly decreased compared with saline-treated mice. Thus, body weights of KK-A^y mice were almost twice as much as those of C57BL/6J mice. Furthermore, liver weights in KK-A^y mice were increased 1.7-fold compared to those of C57BL/6J mice.

The data of serum lipids, glucose and insulin are summarized in Supporting Information Table S1. At 13 weeks of age, the average serum levels of triglyceride, total cholesterol, free fatty acid and insulin of KK-A^y mice with AOM treatment were 484.1 \pm 106.1 mg/dL (mean \pm SD), 101.6 \pm 12.5 mg/dL, 1796 \pm 493 μ Eq/L and 10.1 \pm 3.9 μ g/mL, respectively. These levels of KK-A^y mice with AOM treatment were significantly higher ($p < 0.01$) than those of C57BL/6J mice with AOM treatment. At 19 weeks of age, the average serum levels of triglyceride, total cholesterol, free fatty acid, glucose and insulin of KK-A^y mice with AOM treatment were also significantly higher ($p < 0.01$) than those of C57BL/6J mice with AOM treatment.

As shown in Figures 1a and 1b, the subcutaneous area (indicated in yellow) and visceral area (in red) of abdominal fat in KK-A^y and C57BL/6J mice with AOM treatment could be distinguished in CT images, and subcutaneous, visceral and total amount of fat tissue were calculated individually by instrumental software. The values in all cases were significantly increased ($p < 0.01$) in KK-A^y mice compared with those of C57BL/6J mice at 13 and 19 weeks of age (Supporting Information Table S2). Notably, a marked increase was observed in the visceral fat of KK-A^y mice, such as 7.6 \pm 1.1 g/mouse (at 13 weeks of age) and 11.3 \pm 2.3 g/mouse (at 19 weeks of age).

Histopathological examination of visceral adipose tissue clearly showed enlargement of adipocytes (Figs. 1c and 1d), which was confirmed by quantification of the number of adipocyte nuclei observed in the field of fat tissue in KK-A^y mice (Fig. 1e). Immunohistochemical examination of adipose tissue with F4/80 antibody showed a 2.3-fold increase of infiltrated macrophages into the adipose tissue in KK-A^y mice compared with those of C57BL/6J mice (Fig. 1f). Furthermore, liver steatosis, hypertrophy of pancreatic islets and fatty infiltration in the pancreas were observed in KK-A^y mice (data not shown).

Levels of adipocytokines in serum and visceral fat tissue

Table 1 summarizes data on serum adipocytokine levels in KK-*A^y* and C57BL/6J mice at 13 weeks and 19 weeks. IL-6 and leptin were significantly increased ($p < 0.01$) in KK-*A^y* mice compared with C57BL/6J mice with AOM treatment at 13 weeks and 19 weeks. At 13 weeks, Pai-1 and resistin

were significantly increased in KK-*A^y* mice compared with C57BL/6J mice. Meanwhile, at 19 weeks, Pai-1 and resistin had tendency to increase in KK-*A^y* mice compared with C57BL/6J mice. Serum TNF- α level was not significantly different between KK-*A^y* mice and C57BL/6J mice.

Figures 2a (13 weeks) and 2c (19 weeks) show expression levels of adipocytokines in visceral fat tissue, and MCP-1, Pai-1, TNF- α and leptin were significantly increased in KK-*A^y* mice at 13 weeks and 19 weeks compared with C57BL/6J mice with AOM treatment. Significant increase of IL-6 level was observed at 13 weeks, but not 19 weeks. The level of adiponectin was decreased 40% ($p < 0.05$) at 19 weeks, which was not significant at 13 weeks (Figs. 2b and 2d). Expression levels of Ob-Rbl in the colon of KK-*A^y* and C57BL/6J mice with AOM treatment at 19 weeks were measured by real-time PCR, and it was found that the levels were slightly lower in KK-*A^y* mice ($n = 7$) than in C57BL/6J mice ($n = 7$), but the differences were not statistically significant.

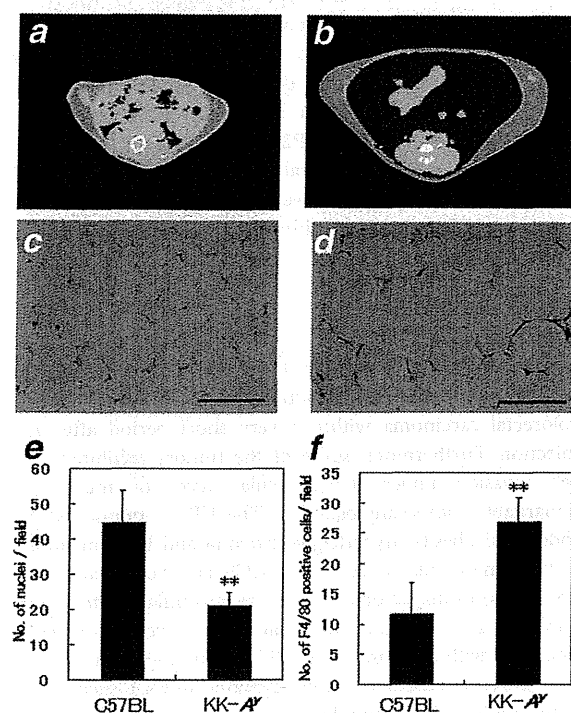


Figure 1. Obese features observed in KK-*A^y* mice at 13 weeks. Abdominal fat scanned by micro-CT was reconstructed to axial images (yellow represents subcutaneous fat and red represents visceral fat) for C57BL/6J (a) and KK-*A^y* mice (b). Histopathological sections of visceral fat in C57BL/6J (c) and KK-*A^y* mice (d) are also shown. Numbers of nuclei of fat cells (e) and F4/80 positive cells (f) were counted per microscopical field in visceral fat tissue as mentioned in Material and Methods section. Data are means \pm SE. Bar represent 200 μ m. ** $p < 0.01$ vs. C57BL/6J mice.

Increased colorectal carcinogenesis in KK-*A^y* mice

All KK-*A^y* mice and C57BL/6J mice developed ACF in the colon and rectum at 13 weeks with AOM treatment (Table 2). In spite of the treatment of both mice with AOM at the same dosage (200 μ g/mouse [≈ 10 mg/kg], once weekly for 3 weeks), induction of colorectal ACF was much greater in KK-*A^y* mice compared to C57BL/6J mice. The number of total ACF in KK-*A^y* mice was 69.6 ± 12.9 /mouse, which was almost 8 times higher than that in C57BL/6J mice. Significant numbers of colorectal ACF were observed in all portions of the colorectum in KK-*A^y* mice, but they were most abundant in the distal portion. The size of aberrant crypts did not differ between C57BL/6J and KK-*A^y* mice. Saline-treated KK-*A^y* and C57BL/6J mice did not develop colorectal ACF.

All of the KK-*A^y* mice developed colorectal tumors by AOM administration at great numbers compared with C57BL/6J mice (Table 3). Most colorectal tumors were distributed in the middle-distal portion. The color of colorectal tumors observed in KK-*A^y* mice was quite different from that observed in C57BL/6J mice, and the color of colorectal tumors in KK-*A^y* mice was intense red (Fig. 3a). Histopathological examination revealed AOM-induced colorectal tumors to be adenomas or adenocarcinomas. Table 3 summarizes

Table 1. Serum adipocytokine levels in KK-*A^y* and C57BL/6J mice treated with AOM

Adipocytokines	13 week-old		19 week-old	
	KK- <i>A^y</i>	C57BL	KK- <i>A^y</i>	C57BL
IL-6 (pg/mL)	40.4 \pm 21.5**	15.9 \pm 2.5	86.0 \pm 57.9**	24.0 \pm 6.6
Leptin (ng/mL)	34.5 \pm 15.2**	1.5 \pm 0.6	40.1 \pm 13.4**	3.0 \pm 2.9
Pai-1 (ng/mL)	3.8 \pm 1.2*	2.3 \pm 1.1	5.6 \pm 1.8	4.1 \pm 0.9
Resistin (ng/mL)	3.6 \pm 0.3**	2.5 \pm 0.2	4.3 \pm 1.3	3.1 \pm 1.0
TNF- α (pg/mL)	14.7 \pm 0.7	13.4 \pm 0.3	16.6 \pm 2.1	16.4 \pm 3.6

Data are mean \pm SD. Significantly different from the C57BL/6J mice at * $p < 0.05$, ** $p < 0.01$, respectively.

data on incidence and multiplicity. The incidence of adenoma was 84% and of adenocarcinoma was 88% in KK-*A*^Y mice, and these values were significantly higher than those of C57BL/6J mice (8% and 4%, respectively). The number of adenoma and adenocarcinoma were 3.20 ± 3.18 and 4.60 ± 4.60 in KK-*A*^Y mice, respectively, and were significantly higher than those of C57BL/6J mice. All adenocarcinomas in both KK-*A*^Y mice and C57BL/6J mice were characterized by tubular structures lined by tall columnar epithelium with scant cytoplasm and enlarged euchromatic nuclei. Particularly in KK-*A*^Y mice, remarkable tumor angiogenesis, hemorrhage and vasodilation were characteristically observed near the surfaces of adenocarcinoma (Fig. 3*b*). Moreover, invasive growth reaching into muscular layer of mucosa was found in 4 out of 22 KK-*A*^Y mice (Fig. 3*c*). Meanwhile, such lesions were not observed in C57BL/6J mice. Metastatic lesions in

the liver and other tissues were not observed in KK-*A*^Y mice with colon adenocarcinoma. Saline-treated KK-*A*^Y and C57BL/6J mice did not develop colorectal tumors.

To clarify the factors which have an effect on high susceptibility of KK-*A*^Y mice to AOM-induced colorectal ACF/tumor development, length of crypt and cells/crypt in the colon were analyzed. The length of colon crypt was longer and the number of cells/crypt was higher in KK-*A*^Y mice with AOM treatment compared with C57BL/6J mice with AOM treatment at 19 weeks (Supporting Information Table S3). Other factors such as difference of AOM metabolism were also evaluated between KK-*A*^Y and C57BL/6J mice. To this end, activities of CYP2E1,²⁴ the main enzyme that metabolizes AOM, were calculated and similar CYP2E1 activities were observed between KK-*A*^Y and C57BL/6J mice (Supporting Information Table S4).

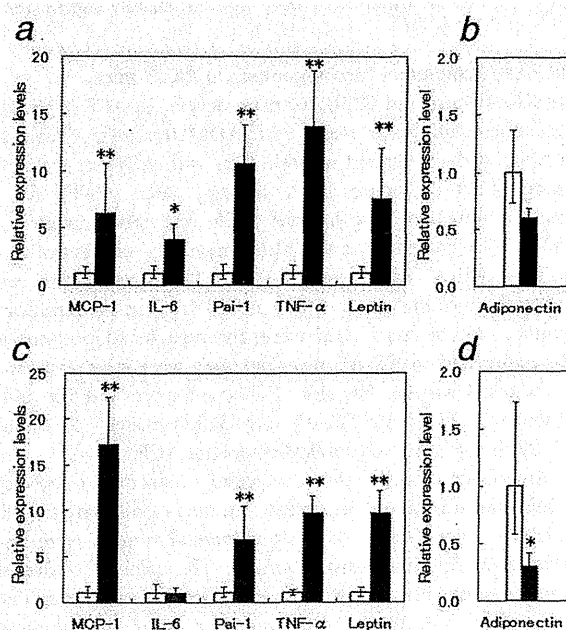


Figure 2. Relative expression levels of adipocytokine mRNA in visceral fat tissue of KK-*A*^Y and C57BL/6J mice treated with AOM. RT-PCR analysis was performed to obtain MCP-1, IL-6, Pai-1, TNF- α , leptin and adiponectin mRNA expression levels at 13 weeks (a and b) and 19 weeks (c and d). GAPDH mRNA was used to normalize the data. KK-*A*^Y mice (■); C57BL/6J mice (□). Data are means \pm SE. * $p < 0.05$, ** $p < 0.01$ vs. C57BL/6J mice.

Discussion

In this study, obese diabetic KK-*A*^Y mice were found to be highly susceptible to induction of ACF, and developed colorectal carcinoma within a very short period after AOM injection. Furthermore, some of the tumors exhibited cancer cell invasion under the muscular layer of mucosa and remarkable tumor angiogenesis. The KK-*A*^Y mouse exhibited abdominal obesity, hypertriglyceridemia and hyperinsulinemia at the time-points of colorectal ACF (13 weeks) and cancer (19 weeks) examinations, and serum pro-inflammatory adipocytokines such as IL-6, leptin and Pai-1 were also elevated compared with those in lean C57BL/6J mice. Moreover, expression of pro-inflammatory adipocytokine mRNA such as IL-6, leptin, MCP-1, Pai-1 and TNF- α was significantly increased in the visceral fat tissue; in contrast, adiponectin was decreased as reported previously.^{21,25,26}

The number of ACF/mouse developed in KK-*A*^Y mice (≈ 70 /mouse) by AOM (200 μ g/mouse [≈ 10 mg/kg], once weekly for 3 weeks) seems to be higher than the number of ACF/mouse developed in other obese mice, *ob/ob* mice or *db/db* mice treated with AOM. It has been reported that injection of AOM (5 mg/kg, once weekly for 4 weeks) to *ob/ob* and *db/db* mice resulted in ≈ 15 colorectal ACF/mouse, and to lean littermates, C57BL, resulted in ≈ 6 ACF/mouse.⁶ High dose of AOM (15 mg/kg, once weekly for 5 weeks) to *db/db* mice were reported to be ≈ 30 ACF/mouse, and ≈ 16 ACF/mouse in their lean litter mates.²⁷ Up to now, studies covering colorectal cancer development in *ob/ob* or *db/db* mice

Table 2. Development of colorectal ACF in KK-*A*^Y and C57BL/6J mice treated with AOM

Mice	No. of mice with ACF	No. of ACF/colorectum					Total	Mean no. of ACFs/focus
		Proximal	Middle	Distal	Rectum			
C57BL	10/10	0.2 ± 0.4	1.6 ± 1.5	4.2 ± 1.0	3.2 ± 0.9	9.2 ± 1.3	1.8 ± 0.3	
KK- <i>A</i> ^Y	10/10	$12.3 \pm 11.4^{**}$	$20.3 \pm 9.0^{**}$	$30.3 \pm 2.3^{**}$	$6.7 \pm 1.0^*$	$69.6 \pm 12.9^{**}$	1.6 ± 0.2	

Data are mean \pm SD. Significantly different from the C57BL/6J mice at * $p < 0.05$, ** $p < 0.01$, respectively.

Table 3. Incidence and multiplicity of colorectal tumors in KK-*A^y* and C57BL/6J mice treated with AOM

Mice	No. of mice	No. of mice with tumors (%)			No. of tumors/mouse		
		Adenoma	Adenocarcinoma	Total	Adenoma	Adenocarcinoma	Total
C57BL	25	2 (8)	1 (4)	3 (12)	0.04 ± 0.20	0.08 ± 0.28	0.12 ± 0.33
KK- <i>A^y</i>	25	21 (84)**	22 (88)**	25 (100)**	3.20 ± 3.18**	4.60 ± 4.60**	7.80 ± 7.20**

Data are mean ± SD. Significantly different from the C57BL/6J mice at ***p* < 0.01.

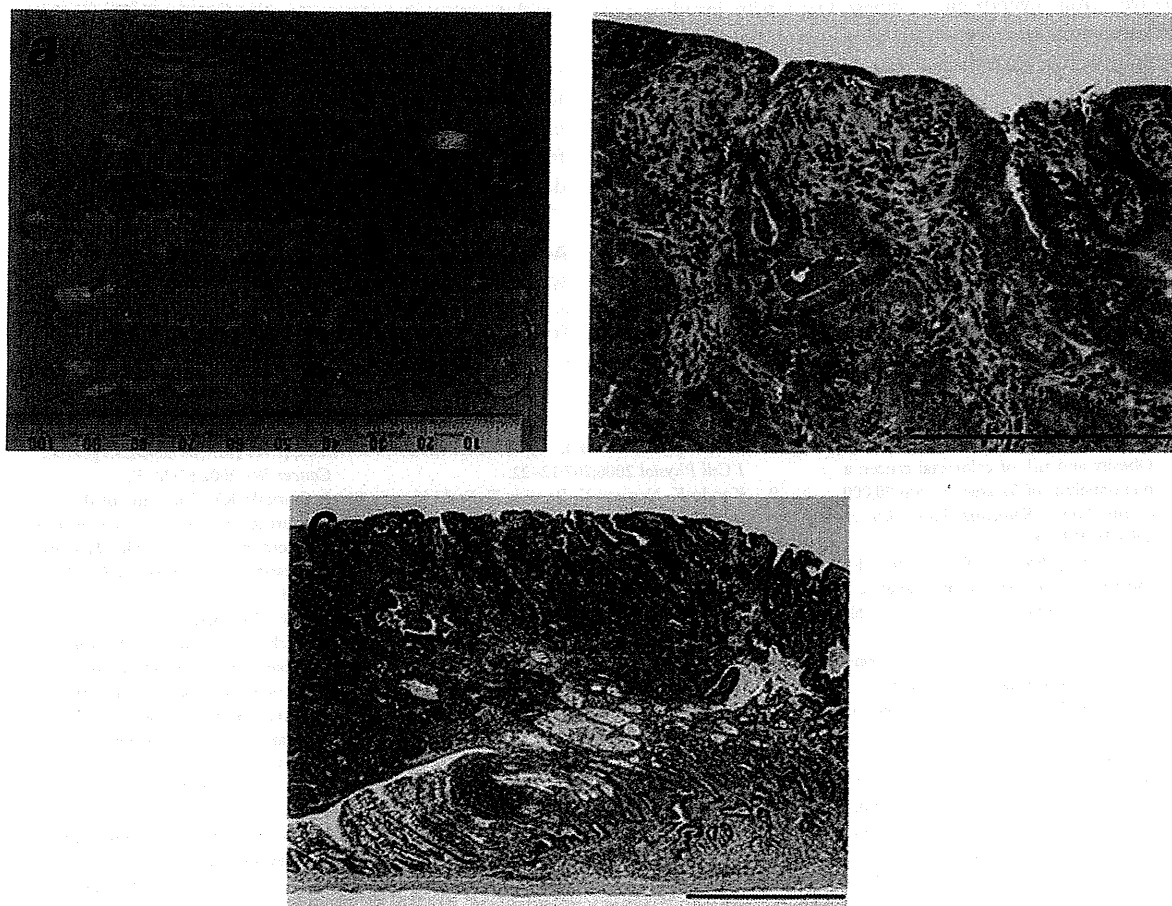


Figure 3. Macroscopic and histopathological analysis of colorectal tumors. (a) Representative macroscopic features of colorectal tumors developing in KK-*A^y* (lower three colorectal segments) and C57BL/6J mice (upper three colorectal segments) with a ruler. (b) Representative adenocarcinoma in KK-*A^y* mouse. Bar represent 200 μ m. (c) Representative histopathologic section of adenocarcinoma showing a neck and body in KK-*A^y* mouse. Bar represent 500 μ m.

have not been reported. In our study, we found that colorectal cancer developed in KK-*A^y* mice within a short period.

In well-established AOM-induced colorectal cancer study, generally it will take almost one year after 6 weekly AOM injection to develop 50–60% of colorectal cancer incidence in C57BL/6J mice.²⁸ Our models developed cancer within 20 weeks after AOM injection. In addition, it is worthwhile to mention that some tumors developing in KK-*A^y* mice showed

cancer cell invasion under the muscular layer of mucosa, which are rarely observed in AOM-induced colorectal cancer in other mice strain.²⁹ Other characteristic features of developed colorectal cancer were its redness. Microscopic examination revealed much vessel formation and vasodilation, account for the redness of the lesions. One mechanism for enhanced tumor angiogenesis could be explained by the presence of elevated leptin and Pai-1 (Fig. 2), which promote

angiogenic signaling.^{8,30} Leptin binding to Ob-Rb1 induces STAT3 binding and resulted in induction of angiogenic factors, such as vascular endothelial growth factor (VEGF).⁸ PAI-1 maintains extracellular matrix integrity required for endothelial cell differentiation.³¹ Moreover, the expression and secretion of VEGF is insulin-dependent, and the circulating VEGF levels are increased in hyperinsulinemic state.³²

Increased serum levels of pro-inflammatory cytokines such as Pai-1 and IL-6 or decreased adiponectin level could be derived from hypertrophic adipose tissue with invading pro-inflammatory monocyte such as activated macrophages.³³ In human beings, such a disruption of adipocytokine production is also observed in metabolic syndrome patients,^{34,35} and changes of adipocytokine balance could be a clue to clarify the relationship between obesity and colorectal cancer. For instance, it has been demonstrated that TNF- α signaling blockade suppresses colorectal carcinogenesis in AOM-DSS treated mice, wherein high TNF- α expressions were observed.³⁴ Treatment of *Apc*-deficient mice with Pai-1 inhibitor suppressed intestinal polyp formation in which high Pai-1 expressions were observed.²² In contrast, adiponectin-knockout mice

fed a high-fat diet increased the number of AOM-induced colon ACF and adenocarcinoma.³⁵ These papers support the idea that high-expression levels of adipocytokines, except for adiponectin, affect the formation of colon ACF and adenocarcinoma as observed in this study. Further investigation using TNF- α blocker, Pai-1 inhibitor and adiponectin stimulator in this novel mouse model could clarify the relationship between obesity and colorectal cancer.

In conclusion, this studies indicated that KK-A^y mice exhibiting a metabolic syndrome profile are highly susceptible to AOM-induced colorectal ACF/tumor development, with occurrence of tumors within a short period. Thus, the KK-A^y mouse could be a useful animal model for human obesity-associated cancer, to clarify important links between factors for the metabolic syndrome and colorectal cancer development.

Acknowledgements

We thank Ms. Misako Iio (University of Shizuoka) for technical supports. S.T. is presently the recipient of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research.

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Increase of oxidant-related triglycerides and phosphatidylcholines in serum and small intestinal mucosa during development of intestinal polyp formation in Min mice

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(Received June 23, 2010/Revised August 24, 2010/Accepted September 12, 2010/Accepted manuscript online September 21, 2010/Article first published online October 14, 2010)

Recent epidemiological studies have shown a positive association of a high-fat diet with the risk of colon cancer. Indeed, increments in the serum levels of triglycerides (TG) and cholesterols are positively related with colon carcinogenesis. We previously reported that an age-dependent hyperlipidemic state is characteristic of Min mice, an animal model for human familial adenomatous polyposis (FAP). However, qualitative and quantitative changes of lipid metabolism are poorly understood in this state. Here, we provide detailed analysis of serum lipids in Min mice using reverse-phased liquid chromatography/electrospray ionization mass spectrometry (RPLC/ESI-MS). We also demonstrate local analysis of lipid droplets in the villi of the small intestine using laser capture microdissection and a sensitive chip-based nanoESI-MS system. As a result, oxidized phosphatidylcholines (PC) such as aldehyde and carboxylic acid types were increased, even at an early stage of intestinal polyp formation in serum. In addition, hydroperoxidizable TG precursors containing linoleic acid (18:2n-6) were deposited at the tip of the villi with aging, and these hydroperoxidized TG were also increased in serum. Meanwhile, increments of the oxidizable TG precursors in serum and small intestinal mucosa were suppressed by treatment with pitavastatin, a novel third generation lipophilic statin. These results suggest that quantitative and qualitative lipid changes such as hydroperoxidizable TG precursors are important in the course of intestinal polyp formation and oxidative stress might lead to the development of intestinal polyp formation in Min mice. (*Cancer Sci* 2011; 102: 79–87)

The incidence and mortality of colon cancer, which is associated with obesity, a high-fat diet and hyperlipidemia according to several epidemiological studies, has increased in developed countries.^(1–5) We previously reported an age-dependent hyperlipidemic state in *adenomatous polyposis coli* (*Apc*)-deficient Min and *Apc*¹³⁰⁹ mice, animal models of familial adenomatous polyposis (FAP).^(6–8) Min mice develop large numbers of intestinal polyps due to truncation mutation in both alleles of the *Apc* gene, leading to activation of Wnt signaling to promote cell growth, which is increased by consumption of a high-fat diet.⁽⁹⁾

Although the direct link between *Apc*-deficiency and hyperlipidemia is yet to be clarified, it is notable that serum triglyceride (TG) levels in Min mice are almost 10 times higher than those observed in wild-type littermates (C57BL/6J) at 20 weeks of age, even though both mice were fed a non-high-fat diet, AIN-76A, including corn oil (5% of total components).⁽⁶⁾ Both groups of mice ate almost the same amount of diet, but the mean

bodyweight was 16% lower in Min mice compared with that of their wild-type littermates at 20 weeks of age, which might be due to the development of intestinal polyps. Consumption of a high-fat diet is associated with hyperlipidemia and leading obesity.⁽¹⁰⁾ However, Min mice featured hyperlipidemia without a high fat-diet intake or obesity. The reasons for the pathology is correlated with decreased mRNA expression levels of lipoprotein lipase (LPL), which hydrolyzes TG into free fatty acids and monoglyceride, in the liver and small intestine.^(11,12) We demonstrated that induction of LPL mRNA by peroxisome proliferator-activated receptor (PPAR)- α and - γ agonists and selective LPL-inducing agent, NO-1886, which lacks potential for activating the PPAR pathways, suppressed the hyperlipidemic status, steatosis of the liver and intestinal polyp formation.^(6–8) In addition, we indicated that a number of large lipid droplets were found in the surface epithelial cells of small intestinal polyps by Oil-red O staining and electron microscopy.⁽¹³⁾ These results suggest that lipid metabolism changes might play important roles in intestinal polyp formation, but the quantitative and qualitative changes have not been well defined in the serum and small intestine.

In this study, we examined a detailed analysis of serum lipids in Min mice using electrospray ionization-mass spectrometry (ESI-MS) coupled with reverse-phased ultra-performance liquid chromatography (UPLC).^(14,15) Moreover, lipid droplets in the villi of the small intestine were collected by laser microdissection (LMD) under direct microscopic visualization and analyzed using a sensitive chip-based nanoESI-MS system by neutral loss scanning of identical fatty acyl groups from individual TG molecular species and precursor ion scanning of phosphoryl choline from individual phosphatidylcholine (PC) molecular species.^(16–22) The possible lipid markers for development of intestinal polyps in Min mice are discussed. Furthermore, the relationship between the markers and polyp formation in Min mice are demonstrated by administration of pitavastatin, a novel 3-hydroxy-3-methylglutaryl coenzyme-A (HMGCoA) reductase inhibitor, which possesses pleiotropic functions including anti-inflammation and anti-oxidant functions.^(23–26)

Materials and Methods

Animals. Male C57BL/6-*Apc*^{Min/+} mice (Min mice) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA)

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at 6 weeks of age and genotyped as previously reported.⁽²⁷⁾ Heterozygotes of Min strain and wild-type (C57BL/6J) mice were acclimated to laboratory conditions for 1 week. Less than five mice were housed per plastic cage with sterilized softwood chips as bedding in a barrier-sustained animal room at 24°C ± 2°C and 55% humidity on a 12 h light/dark cycle.

To investigate lipid metabolism changes, male Min mice ($n = 9$) and wild-type mice ($n = 6$) at 5 weeks of age were given AIN-76A powdered basal diet (CLEA Japan, Tokyo, Japan). Min mice ($n = 3$) and wild-type mice ($n = 2$) were killed at 10, 15 and 20 weeks of age. To examine the effects of pitavastatin ([+]-monocalcium bis [3*R*,5*S*,6*E*]-7-[2-cyclopropyl-4-(4-fluorophenyl)-3-quinolyl]-3,5-dihydroxy-6-heptenoate), which was kindly provided by Kowa Pharmaceutical Co., Ltd (Aichi, Japan), male Min mice at 6 weeks of age were given pitavastatin, which was mixed well at concentrations of 40 p.p.m. for 14 weeks in AIN-76A. Min mice with pitavastatin treatment ($n = 3$) and with pitavastatin untreated ($n = 3$) were killed at 20 weeks of age. Food and water were available *ad libitum*. The mice were observed daily for clinical signs and mortality. Body-weights and food consumption were measured weekly. 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.

Materials. All solvents for HPLC or MS grade were purchased from Wako Pure Chemicals (Osaka, Japan). Deionized water was obtained from a Milli-Q water system (Millipore, Milford, MA, USA).

Extraction and isolation of serum and small intestinal mucosa lipids. Total lipids from serum and small intestinal mucosa were extracted using Bligh and Dyer's method in the following procedure:⁽²⁸⁾ serum (75 μ L) and small intestinal mucosa in the proximal segments (50 mg) removed by scraping were individually homogenized with 6 mL chloroform/methanol (1:2) for 10 strokes and left for 1 h at room temperature. In this process, 1 nmol of sphingomyelin (SM) (d18:1-12:0) was added as an internal standard. Each phase separation was achieved by adding 2 mL chloroform and 2 mL water. After vortexing, the mixture was centrifuged at 1000*g* for 10 min. The bottom organic layer containing the total lipid extract was collected and dried under a gentle stream of nitrogen, and then dissolved by 200 μ L chloroform-methanol (2:1) and normalized to the serum volume and intestinal mucosa weight.

Laser capture microdissection of the small intestine. The intestinal tracts of male Min mice at 10 and 20 weeks old ($n = 3$) were removed and separated into the small intestine. Each small intestine was divided into the proximal segment (4 cm in length), and then opened longitudinally and fixed flat between sheets of filter paper in 10% buffered formalin for further Oil-red O

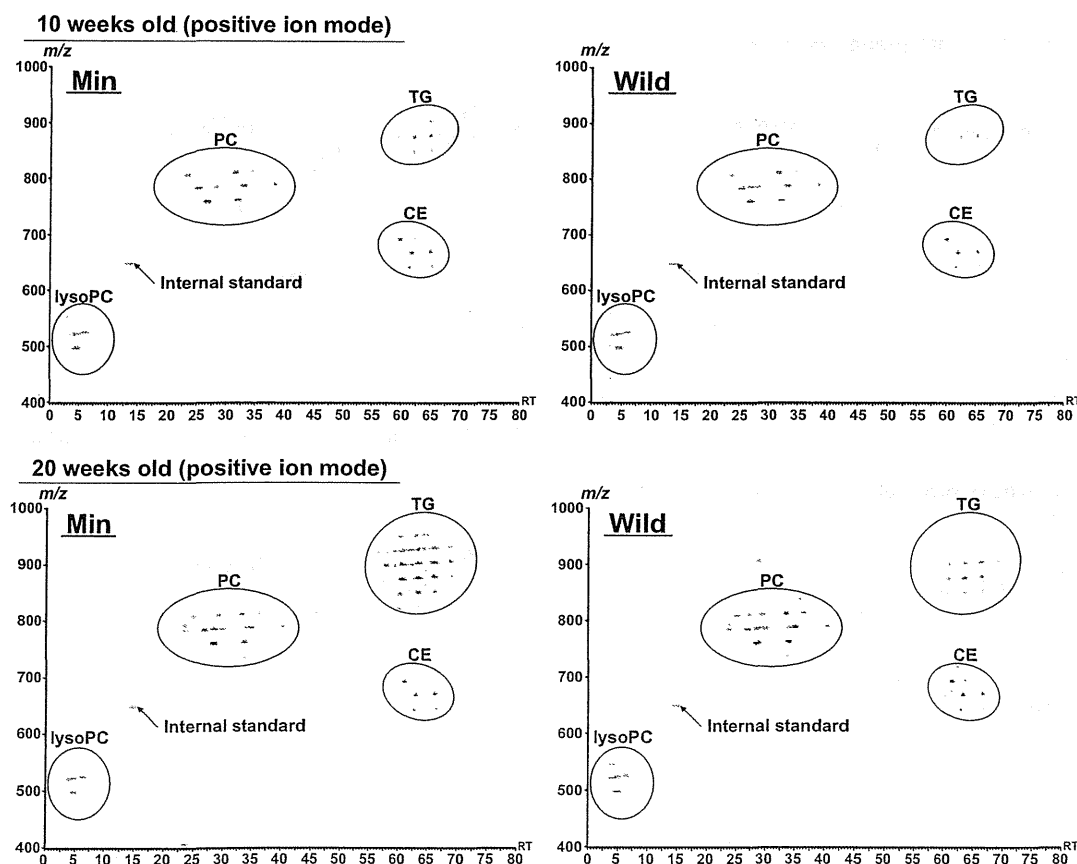


Fig. 1. 2-D profiling analysis of serum lipids from Min mice by reverse-phased liquid chromatography/electrospray ionization mass spectrometry (RPLC/ESI-MS) in positive ion mode. Each of the lipid classes was detected in the following order: lysophosphatidylcholine (lysoPC) > phosphatidylcholine (PC) > triglyceride (TG), cholesteryl ester (CE). The TG of Min mice were increased at 10 weeks of age and highly elevated at 20 weeks of age. 2-D lipid maps were constructed with X (retention time) and Y (m/z value) axes, and the intensity of these peaks was adjusted by color density spots.

staining and LMD.⁽⁶⁾ Lipid droplets of villi in the small intestine were observed using modified Oil-red O staining method for confirmation of the locus.⁽²⁹⁾ In brief, each frozen section as Swiss rolls was treated with 60% isopropanol for 1 min, and stained with Oil-red solution for 15 min at room temperature. The dye was then removed, and the section was washed with PBS and 60% isopropanol each for 2 min. For local analysis of the villi, each frozen section was mounted on DIRECTOR LMD slide (AMR Inc., Tokyo, Japan), and lipid droplets from the tip and basis of the villi were collected by Leica LMD system, LMD 6000 (Leica Microsystems, Wetzlar, Germany) with a pulsed 355-nm diode laser and individually extracted by methanol.⁽²²⁾ The correction intensity in each graph was calculated by the ratio of TG to the endogenous standard (16:0-18:2 PC).

Reverse-phased liquid chromatography/ESI-MS (RPLC/ESI-MS) conditions. The RPLC/ESI-MS analysis was performed using a quadrupole/time-of-flight hybrid mass spectrometer, Q-TOF micro (Waters Corporation, Milford, MA, USA) with an ACQUITY UPLC system (Waters Corporation).⁽¹⁴⁾ The scan range of the instrument was set at m/z 200–1100, the scan duration of MS and MS/MS was at 0.5 s and the collision gas used for the MS/MS experiments was at 7.5×10^5 mbar (argon). The capillary voltage in positive ion mode was set at 3.5 kV, cone voltage at 30 V and collision energy of MS/MS at 30 V, whereas capillary voltage was at -2.5 kV, cone voltage at -30 V and collision energy of MS/MS at -30 V in negative ion mode.

The RPLC separation was achieved using an ACQUITY UPLC BEH C18 column (150 × 1.0 mm inner diameter [i.d.], Waters Corporation) at 45°C. Two microlitre of total lipids normalized to serum volume was individually injected. The mobile phase was acetonitrile/methanol/water : 19/19/2 (0.1% formic acid + 0.028% ammonia) (A) and isopropanol (0.1% formic acid + 0.028% ammonia) (B), and the composition was produced by mixing these solvents. The gradient consisted of holding solvent (A/B : 90/10) for 7.5 min, then linearly converting to solvent (A/B : 70/30) for 32.5 min and finally linearly converting solvent (A/B : 40/60) for 50 min. The mobile phase was pumped at a flow rate of 40–50 $\mu\text{L}/\text{min}$. The MS data processing was applied by Mass++ software (<http://masspp.jp/>) to detect each chromatogram peak with quantitative accuracy. The correction intensity in each graph was calculated by the ratio of TG or phosphatidylcholine (PC) to the internal standard.

Multiple reaction monitoring (MRM) conditions. The MRM analysis was performed using a quadrupole-linear ion trap hybrid mass spectrometer, 4000Q TRAP (AB SCIEX, Foster City, CA, USA) with the same ACQUITY UPLC system as previously reported.⁽¹⁵⁾ Ten microlitre of total lipids normalized to serum volume was individually injected. The mobile phase was acetonitrile/methanol/water : 2/2/1 (0.1% formic acid + 0.028% ammonia) (A) and isopropanol (0.1% formic acid + 0.028% ammonia) (B), and the composition was produced by mixing these solvents. The gradient consisted of holding solvent

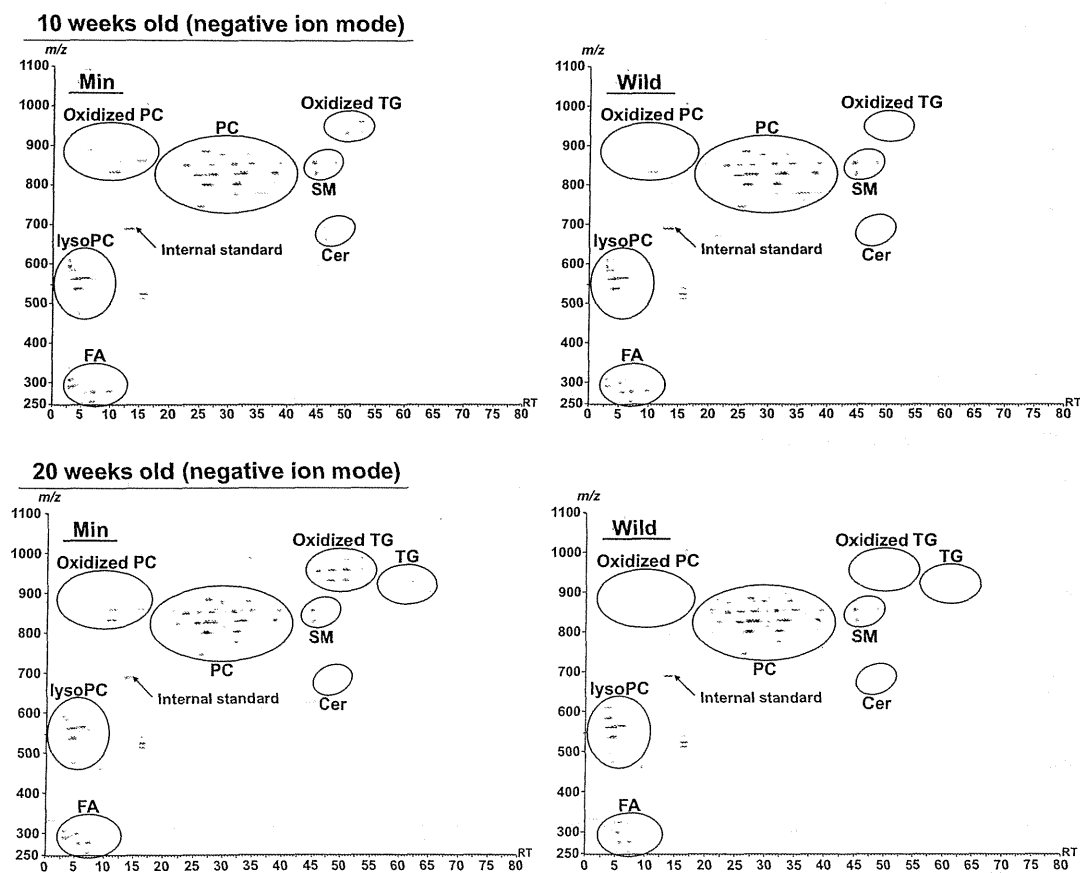


Fig. 2. 2-D profiling analysis of serum lipids from Min mice by reverse-phased liquid chromatography/electrospray ionization mass spectrometry (RPLC/ESI-MS) in negative ion mode. Each of the lipid classes was detected in the following order: oxidized phosphatidylcholine (PC), lysophosphatidylcholine (lysoPC), fatty acid (FA) > PC > sphingomyelin (SM), ceramide (Cer), oxidized triglyceride (TG) > TG. Oxidized PC and TG of Min mice were highly increased even at 10 weeks of age and highly elevated at 20 weeks of age.

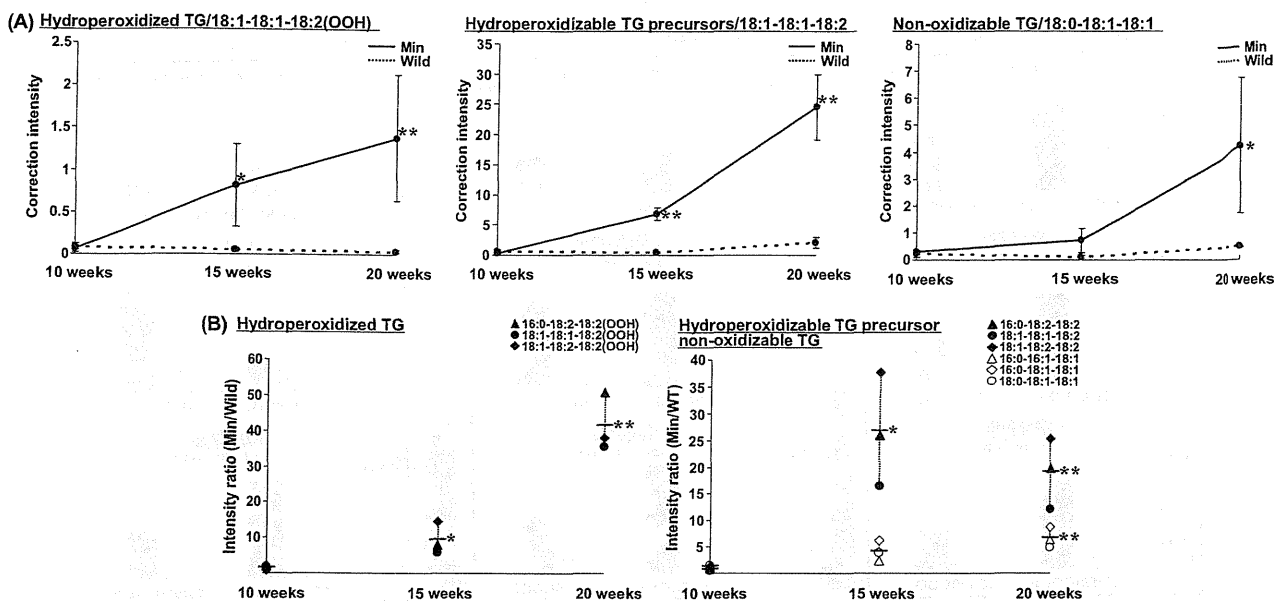


Fig. 3. Quantitative and qualitative profiles of serum triglyceride (TG) molecular species of Min mice in the course of intestinal polyp formation. (A) Compared with wild-type mice, hydroperoxidized TG and hydroperoxidizable TG precursors of Min mice were highly increased from 15 weeks of age, whereas non-oxidizable TG of Min mice were somewhat elevated at 20 weeks of age. Values are mean \pm SD ($n = 3$; $*P < 0.05$, $**P < 0.01$ versus correction intensity of Min mice at 10 weeks of age). (B) Regarding ratios of Min mice to wild-type mice in serum TG molecular species, hydroperoxidized TG ratios were elevated in a time-dependent manner, and hydroperoxidized TG of Min mice at 20 weeks of age were 30–50 times larger than those of wild-type mice. Hydroperoxidizable TG precursor ratios were highly increased at 15 weeks of age and then somewhat decreased at 20 weeks of age, whereas non-oxidizable TG ratios were gradually increased in a time-dependent manner. Values are mean \pm SD ($n = 3$; $*P < 0.05$, $**P < 0.01$ versus intensity ratio at 10 weeks of age) and the individual mean values of these TG are indicated by crossbars.

(A/B : 100/0) for 5 min, then linearly converting to solvent (A/B : 50/50) for 20 min and finally holding solvent (A/B : 50/50) for 34 min at a flow rate of 70 $\mu\text{L}/\text{min}$ and column temperature of 30°C.

NanoESI-MS conditions. Chip-based nanoESI-MS analysis was performed using a 4000Q TRAP with chip-based ionization source, TriVersa NanoMate (Advion BioSystems, Ithaca, NY, USA). The ion spray voltage was set at 1.25 kV, gas pressure at 0.3 pound per square inch (psi) and flow rates at 200 nL/min. The scan range was set at m/z 400–1100, declustering potential at 100 V, collision energies at 50–70 V and resolutions at Q1 and Q3, “unit”. The mobile phase composition was chloroform/methanol: 1/2 (0.1% ammonium formate). Total lipids were directly subjected by flow injection and selectivity analyzed by neutral loss scanning of identical fatty acyl groups from individual TG molecular species and precursor ion scanning of phosphorylcholine from individual PC molecular species.^(16–21)

Statistical analysis. The student’s *t*-test was used for statistical analysis. Values with $P < 0.05$ were considered to be statistically significant.

Results

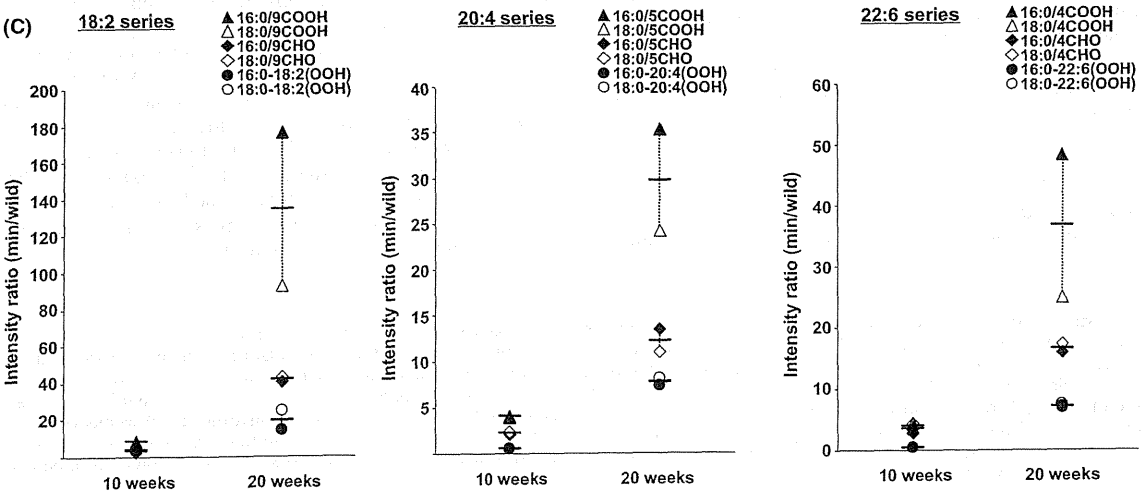
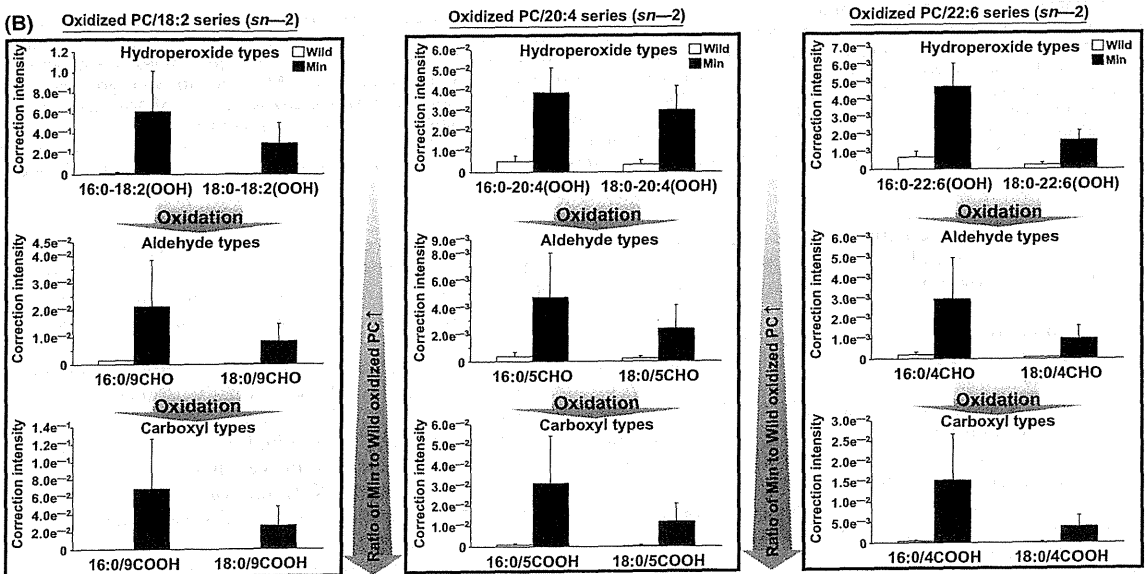
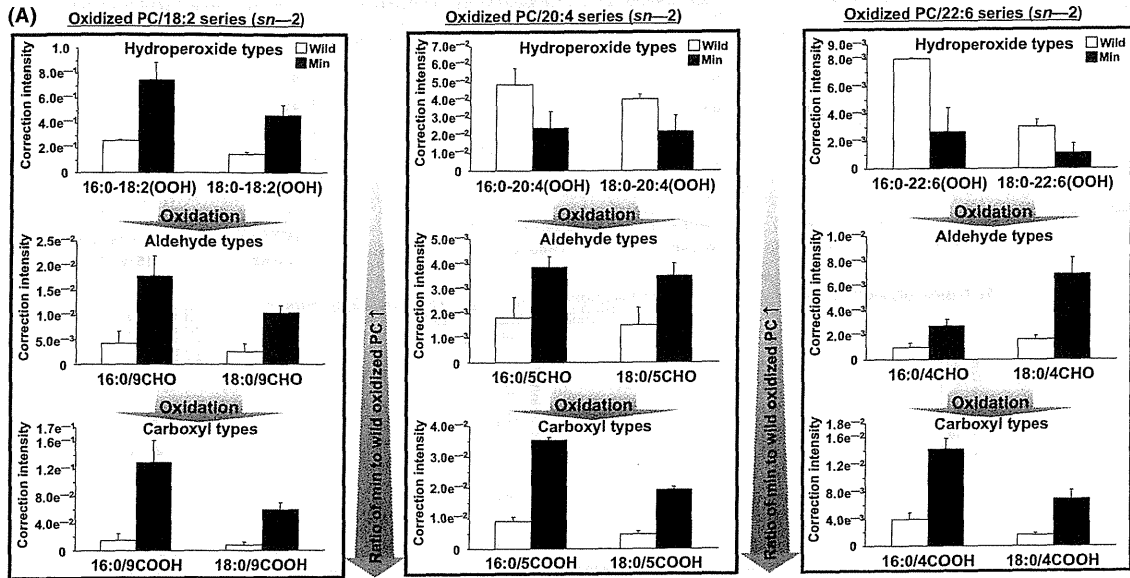
Global analysis of serum lipids from Min mice by 2-D profiling. Intestinal polyp counts in Min mice were obviously

increased with age compared with wild-type mice due to down-regulation of LPL expression. Indeed, the average number of intestinal polyps developed in Min mice was 82.4 ± 8.4 (mean \pm SE). However, none of the wild-type mice developed intestinal polyps.

To further examine the effects on lipid metabolism at the molecular species level, we globally analyzed serum lipids at 10, 15 and 20 weeks of age by RPLC/ESI-MS and then created 2-D lipid maps of the individual precursor ion peaks for searching quantitative and qualitative changes. The 2-D lipid maps were constructed with X (retention time) and Y (m/z value) axes, and the intensity of these peaks was adjusted by color density spots.

As a result, TG increments in Min mice were detected on the map of positive ion mode (55–70 min) even at 10 weeks of age and were markedly found at 20 weeks of age (Fig. 1). In addition, several spots were abundantly observed on the map of negative ion mode (5–15 and 45–55 min) in Min mice compared with wild-type mice (Fig. 2). These distinctive spots were analyzed by MS/MS and respectively identified as oxidized PC and TG, which we first detected from mouse TG in white adipose tissue as previously reported.⁽¹⁴⁾ At 20 weeks of age, these oxidized PC and TG were highly increased in Min mice, suggesting that enhancement of oxidative stress might be caused in the course of intestinal polyp formation.

Fig. 4. Quantitative and qualitative profiles of serum oxidized phosphatidylcholine (PC) molecular species of Min mice in the course of intestinal polyp formation. (A) Oxidized PC derived from peroxidation of polyunsaturated fatty acids (PUFA) such as linoleic acid (18:2n-6), arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3) at the *sn*-2 position were analyzed by multiple reaction monitoring. Aldehyde (CHO) and carboxylic acid (COOH) types of Min mice ($n = 3$) were increased even at 10 weeks of age compared with wild-type mice ($n = 2$). Values are mean \pm SD. (B) In addition to these types, hydroperoxide (OOH) types of Min mice ($n = 3$) were elevated at 20 weeks compared with wild-type mice ($n = 2$). Values are mean \pm SD. (C) The PC ratios of Min mice to wild-type mice were highly increased at 20 weeks of age compared with 10 weeks of age in the following order: carboxylic acid types > aldehyde types > hydroperoxide types. Individual mean values of these types are indicated by crossbars.



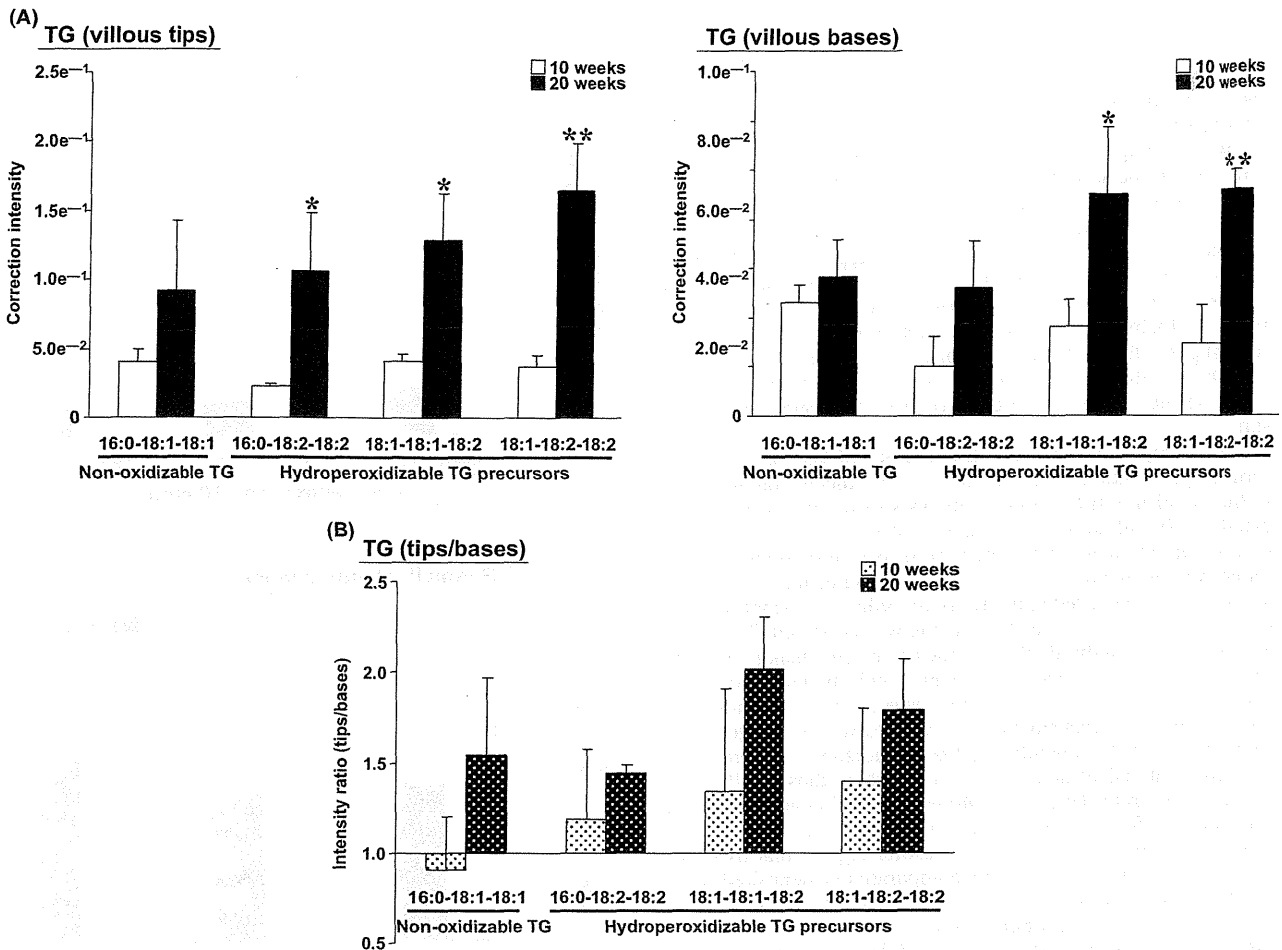


Fig. 5. Local analysis of lipid droplets in Min mice by laser microdissection and the nano-electrospray ionization-mass spectrometry (nano-ESI-MS) system. (A) Hydroperoxidizable triglyceride (TG) precursors at villous tips and bases of Min mice were more increased at 20 weeks of age than 10 weeks of age. Values are mean \pm SD ($n = 3$; * $P < 0.05$, ** $P < 0.01$ versus correction intensity at 10 weeks of age). (B) From hydroperoxidizable TG precursor ratios of villous tips to villous bases, hydroperoxidizable TG precursors of villous tips were 1.5–2 times larger than those of villous bases at 20 weeks of age. Values are mean \pm SD ($n = 3$).

Quantitative and qualitative changes of serum TG molecular species of Min mice in the course of intestinal polyp formation. These spots were more precisely quantified by Mass++, MS data processing software to detect individual chromatogram peaks with quantitative accuracy. In Min mice, serum TG molecular species such as hydroperoxidized (OOH) TG, which were derived from peroxidation of intramolecular linoleic acid (18:2n-6), and hydroperoxidizable TG precursors containing 18:2, which were oxidizable to the hydroperoxidized TG, were increased in a time-dependent manner (Fig. 3A). In particular, hydroperoxidizable TG precursors were highly elevated from 15 weeks of age. Meanwhile, non-oxidizable TG containing saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA) were quantitatively unchanged until 15 weeks of age compared with wild-type mice and somewhat increased at 20 weeks of age. These results suggest that individual TG molecular species were not uniformly elevated, but oxidant-related TG were preferentially increased. As for the TG ratios of Min mice to wild-type mice, hydroperoxidized TG ratios were elevated in a time-dependent manner, and hydroperoxidized TG of Min mice at 20 weeks of age were 30–50 times larger than those of wild-type mice (Fig. 3B). Hydroperoxidizable TG

precursor ratios were highly increased at 15 weeks of age and then somewhat decreased at 20 weeks of age, whereas non-oxidizable TG ratios were gradually elevated in a time-dependent manner. There were significant differences between hydroperoxidized TG ratios and non-oxidizable TG ratios at 20 weeks of age, and between hydroperoxidizable TG precursor ratios and non-oxidizable TG ratios at 15 weeks of age. It seems that these ratios might be effective indications for the pathology of intestinal polyp formation.

Quantitative and qualitative changes of serum PC molecular species of Min mice in the course of intestinal polyp formation. Regarding serum PC molecular species, hydroperoxidizable PC precursors containing 18:2 and non-oxidizable PC containing SFA were quantitatively unchanged between Min and wild-type mice at 10 and 20 weeks of age (Fig. S1). Meanwhile, oxidized PC of Min mice were abundantly observed on the 2-D map, and then a large variety of these oxidized types such as OOH, aldehydes (CHO) and carboxylic acids (COOH) were analyzed in detail by MRM with theoretically expanded data sets as previously reported.⁽¹⁵⁾ These oxidized types were derived from enzymatic and non-enzymatic reactions of peroxidation of polyunsaturated fatty acids (PUFA) such as 18:2,

arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3) at the *sn*-2 position and important in inflammatory biomarkers for the physiological and pathological phenomena.^(30,31) Interestingly, aldehyde and carboxylic acid types derived from peroxidation of 18:2, 20:4 and 22:6 were increased even at 10 weeks of age in Min mice (Fig. 4A), indicating that oxidative stress might occur at the early stage of polyp formation. Besides these aldehyde and carboxylic acid types, hydroperoxide types were highly elevated at 20 weeks in Min mice (Fig. 4B). In addition, PC ratios of Min mice to wild-type mice in these oxidized types were increased at 20 weeks of age compared with 10 weeks of age in the following order: carboxylic acid types > aldehyde types > hydroperoxide types (Fig. 4C). These data support the ideas that Min mice at 20 weeks of age were at a high oxidative stress level and these oxidants might also be effective indications of the exacerbation stage and inflammatory state.

Local analysis of lipid droplets in the villi of Min mice. Because serum lipid contents might be affected by dietary fat absorption in the small intestine, detailed analysis of the villi were investigated at 10 and 20 weeks of age in Min mice by LMD, which permits procurement of the locus from frozen sections of Swiss-rolled middle and distal parts of the small intestines. Lipid accumulation was observed at the tip of the villi in the small intestine with Oil-red O staining and the locus was collected. These lipid mixtures were individually extracted by methanol from the collected tissues and analyzed using a chip-based nanoESI-MS system. As a result, PC molecular species were quantitatively and qualitatively unchanged at 10 and 20 weeks of age in Min mice (Fig. S2). Meanwhile, hydroperoxidizable TG precursors were more abundant at 20 weeks of age than those at 10 weeks of age in Min mice (Fig. 5A). Moreover, the TG at the tip of the villi were 1.5–2.5 times larger than those at the basis at 20 weeks of age (Fig. 5B). These results suggest that hydroperoxidizable TG precursors were comparatively deposited at the tip of the villi with age in Min mice and might be subjected to oxidative stress and inflammation in the small intestine. As discussed previously, hydroperoxidized TG and hydroperoxidizable TG precursors in the serum of Min mice were increased at 20 weeks of age, which might be caused by the increments of hydroperoxidizable TG precursors at the tip of the villi.

Suppression of hydroperoxidizable TG precursor production in the serum and small intestinal mucosa of Min mice by pitavastatin treatment. To examine the relationship between oxidative stress and polyp formation in Min mice, detailed analysis of total lipid extract from the serum and small intestinal mucosa of Min mice treated with pitavastatin at doses of 40 p.p.m. for 14 weeks were investigated by a chip-based nanoESI-MS system. Intestinal polyp formation in the pitavastatin-treated groups was significantly suppressed (the data will be presented in a separate report). The PC molecular species were quantitatively and qualitatively unchanged between the pitavastatin-treated and untreated groups (data not shown). Meanwhile, the intensity ratios of hydroperoxidizable TG precursors to non-oxidizable TG (16:0-18:1-18:1) were significantly decreased in the serum and intestinal mucosa of the pitavastatin-treated groups compared with the untreated groups (Fig. 6). These results suggest that hydroperoxidizable TG precursors are important in developing intestinal polyp formation and hydroperoxidized TG derived from the oxidizable TG precursors might induce oxidative stress and inflammation.

Discussion

In this study, oxidant-related TG and oxidized PC such as aldehyde and carboxylic acid types were increased even at the early stage of intestinal polyp formation in the serum of Min mice. The oxidized PC of Min mice were highly elevated even at

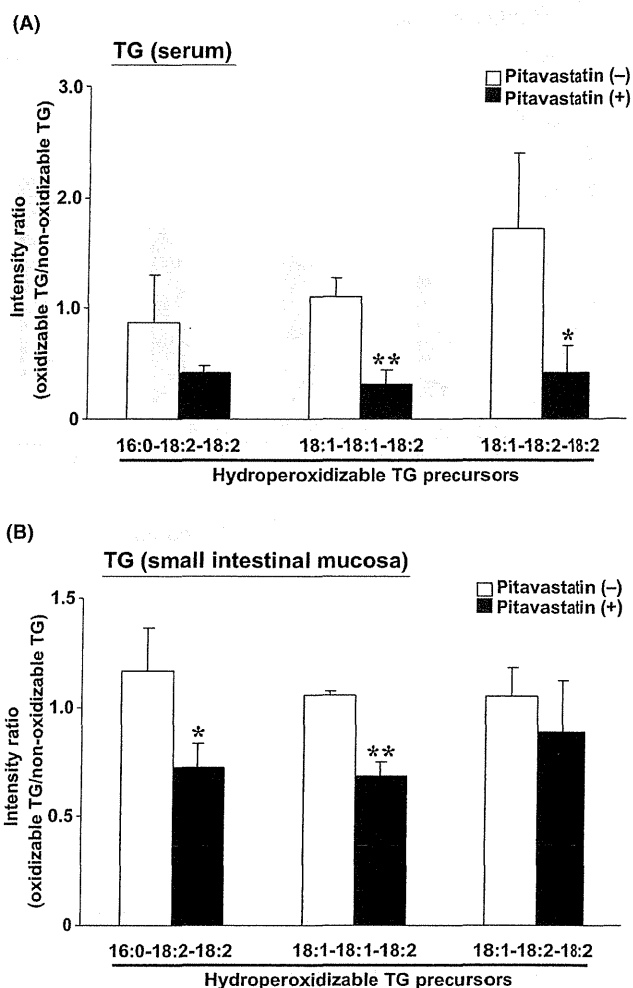


Fig. 6. Suppression of hydroperoxidizable triglyceride (TG) precursor production in serum and the small intestinal mucosa of Min mice by pitavastatin treatment. (A) Intensity ratios of hydroperoxidizable TG precursors to non-oxidizable TG (16:0-18:1-18:1) were significantly decreased in the serum of the pitavastatin-treated groups at doses of 40 p.p.m. for 14 weeks along with suppression of intestinal polyp formation compared with the untreated groups. (B) Similarly, the intensity ratios in the intestinal mucosa of the pitavastatin-treated groups were significantly reduced. Values are mean \pm SD ($n = 3$; * $P < 0.05$, ** $P < 0.01$ versus intensity ratios of the pitavastatin-untreated groups).

10 weeks of age. In particular, hydroperoxidized TG and hydroperoxidizable TG precursors were significantly increased from 15 weeks of age in a time-dependent manner. We previously reported that Min mice had higher levels of 18:2 in plasma compared with wild-type mice by gas-liquid chromatography method.⁽³²⁾ These results suggest that oxidant-related TG in the blood increased in the course of intestinal polyp formation, and these TG might be useful as biological markers for the formation and development of intestinal polyps.

The present study also showed that hydroperoxidizable TG precursors were comparatively deposited at the tip of villi with age in Min mice from our local analysis using LMD and sensitive chip-based nanoESI-MS system. Dietary fat mainly constituted of TG is absorbed at the tip of the villi and accumulates in the cytoplasm of intestinal epithelial cells, but not in stromal cells. The origin of the accumulated lipids seems to be derived

from the contents in the digestive tract, not from blood vessels. Therefore, our detailed analysis of the villi in Min mice is thought to be effective for directly detecting qualitative and quantitative changes. To our knowledge, local lipid analysis of the villi has not yet been reported and accumulation of hydroperoxidizable TG precursors with age was first detected in Min mice. In addition, detailed analysis of serum and the intestinal mucosa in Min mice treated with pitavastatin revealed that hydroperoxidizable TG precursors were significantly decreased along with suppression of intestinal polyp formation. It is speculated that accumulation of hydroperoxidizable TG precursors in the small intestine mucosa leads to an increased serum level of hydroperoxidized TG, and oxidative stress and inflammation might be systemically induced by these oxidized TG. For one reason, the fact that malabsorption of the hydroperoxidizable TG precursors occurs at the tip of the villi, which might evoke a source for increments of hydroperoxidized TG in serum. For another reason, hydroperoxidizable TG precursor increments at the tip of the villi lead to an elevated influx into the general circulation, and hydroperoxidized TG might be abundantly generated by peroxidation of these precursors. Moreover, hydroperoxidized TG derived from these precursors could induce DNA damage, which might link to truncation mutation in both alleles of the *Apc* gene. However, further experiments are needed to sensitively detect oxidized PC and TG from the small

regions of the villi by our local analysis, and to clarify the role of hydroperoxidized TG in intestinal polyp formation.

In conclusion, hydroperoxidizable TG precursors of Min mice in serum and the small intestine mucosa were increased in the course of intestinal polyp formation, and increase of these oxidizable TG precursors was suppressed by pitavastatin. These oxidant-related lipids might be useful as biological indications for the formation and development of intestinal polyps. Furthermore, hydroperoxidized TG, hydroperoxidizable TG precursors and oxidized PC such as aldehyde and carboxylic acid types were highly increased even at 10 weeks of age, and these lipids might be possible markers for early intestinal polyp formation. These results also suggest that qualitative changes of TG and PC are important in the course of intestinal polyp formation and might lead to their development in Min mice.

Acknowledgments

The authors thank Reiko Yajima for technical assistance. This study was performed with the help of Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST).

Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Quantitative and qualitative profiles of serum phosphatidylcholine (PC) molecular species of Min mice in the course of intestinal polyp formation. The PC molecular species in the serum of Min mice ($n = 3$) were quantitatively and qualitatively similar to wild-type mice ($n = 2$). Values are mean \pm SD. The PC containing docosahexaenoic acid (22:6n-3) was not included in the AIN-76A diet and the correction intensities were very small.

Fig. S2. Local analysis of phosphatidylcholine (PC) molecular species at the villous tips and bases of Min mice by laser microdissection and the nano-electrospray ionization-mass spectrometry (nanoESI-MS) system. The PC molecular species at the villous tips and bases of Min mice were quantitatively and qualitatively unchanged at 10 and 20 weeks of age. There was no significant difference between the villous and base values. Values are mean \pm SD ($n = 3$). The PC containing 22:6 was not included in the AIN-76A diet and the intensities were very small.

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Review

Experimental Animal Models of Pancreatic Carcinogenesis for Prevention Studies and Their Relevance to Human Disease

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Received: 1 December 2010; in revised form: 29 December 2010 / Accepted: 26 January 2011 /

Published: 9 February 2011

Abstract: Pancreatic cancer is difficult to cure, so its prevention is very important. For this purpose, animal model studies are necessary to develop effective methods. Injection of *N*-nitrosobis(2-oxopropyl)amine (BOP) into Syrian golden hamsters is known to induce pancreatic ductal adenocarcinomas, the histology of which is similar to human tumors. Moreover, *K-ras* activation by point mutations and *p16* inactivation by aberrant methylation of 5' CpG islands or by homozygous deletions have been frequently observed in common in both the hamster and humans. Thus, this chemical carcinogenesis model has an advantage of histopathological and genetic similarity to human pancreatic cancer, and it is useful to study promotive and suppressive factors. Syrian golden hamsters are in a hyperlipidemic state even under normal dietary conditions, and a ligand of peroxisome proliferator-activated receptor gamma was found to improve the hyperlipidemia and suppress pancreatic carcinogenesis. Chronic inflammation is a known important risk factor, and selective inhibitors of inducible nitric oxide synthase and cyclooxygenase-2 also have protective effects against pancreatic cancer development. Anti-inflammatory and anti-hyperlipidemic agents can thus be considered candidate chemopreventive agents deserving more attention.

Keywords: pancreatic cancer; hyperlipidemia; iNOS; hamster; BOP

1. Introduction

In recent years, pancreatic cancer has increased to become the fifth leading cause of cancer mortality in Japan [1]. Since the five-year-survival rate is very low, elucidation of the mechanisms of pancreatic carcinogenesis and development of prevention methods are important high priority tasks. Factors affecting pancreatic cancer development have been studied using several *in vivo* animal models [2]. Use of *N*-nitrosobis(2-oxopropyl)amine (BOP) in the Syrian golden hamster is known to be unique for development of pancreatic ductal adenocarcinomas, the histology of which is similar to that in human cases [3-6]. In the hamster model, early lesions such as focal hypertrophy, hyperplasia, goblet cell metaplasia, atypical hyperplasia and *in situ* carcinoma sequentially develop in the common duct, pancreatic duct and ductules, but not in acinar cells [7]. Transplacental induction of pancreatic ductal cancer by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and ethanol in Syrian golden hamster is also an interesting model to investigate a synergistic effect of cigarette smoking and alcohol drinking on fetuses [8]. In rats, the azaserine-induced pancreatic cancer model is well-known, but the lesions are acinar cell carcinomas [9]. A nitrosourea amino acid carcinogen, *N*-delta-(*N*-methyl-*N*-nitrosocarbamoyl)-L-ornithine (MNCO), has further been shown to cause pancreatic acinar cell carcinomas in rats [10] and ductal carcinomas in hamsters [11]. Different from in hamsters, BOP mainly induces thyroid gland tumors in rats [12,13] and lung and liver tumors in mice [14]. There is thus a species specificity in the types of pancreatic neoplasm induced in rodents [15-17]. The 7,12-dimethylbenzanthracene (DMBA)-induced pancreatic cancer model can also be employed as a chemical carcinogenesis model in rats [18,19] and mice [20]. In this case, direct implantation of the carcinogen into the head of the pancreas causes tubular complexes in acini and induces pancreatic neoplasms of ductal phenotype in which cytokeratin 19 is expressed [21] and *K-ras* gene mutations are present [22]. Recently, genetically engineered mouse (GEM) models of pancreatic exocrine cancer have been developed and used to elucidate mechanisms of pancreatic carcinogenesis, although the pathology is somewhat different from human cases [23]. Mouse models with pancreas-specific expression of mutant *K-ras* from the embryonic stage frequently develop acinar-to-ductal metaplasia and pancreatic intraductal neoplasms (PanINs), but few pancreatic cancers under normal conditions [24-26]. Additional alterations in tumor-suppressor genes, such as *p16* [27], *p53* [28], *dpc4* [29], and TGF- β receptor II [30], or pancreatitis [31] in the GEM models have been shown to cause quite high incidences of pancreatic cancers. On the other hand, conditional expression of mutant *K-ras* in the adult phase hardly induces PanINs and cancer if without pancreatitis [31]. Transgenic rats that express a mutated Ha- or *K-ras* oncogene regulated by the *Cre/lox* system have also been demonstrated to develop pancreatic ductal carcinomas upon injection of a *Cre*-carrying adenovirus into the pancreatic ducts and acini via the common bile duct [32,33]. In these rat models, mutant *Ras* is conditionally expressed in the pancreas of young adult rats and neoplastic lesions arise in pancreatic duct epithelium, intercalated ducts and centroacinar cells, but not acinar cells [32].

Here, we focus on the BOP-induced pancreatic cancer model in hamsters and discuss its utility for cancer prevention studies.

2. Genetic Alterations in Pancreatic Ductal Carcinomas of Humans and BOP-treated Hamsters

Pancreatic carcinogenesis is known to be a multi-step process involving multiple genetic alterations in humans [34-37] and similar genetic alterations have been found in hamsters [38,39]. Findings for genetic alterations in pancreatic ductal cancers in the two species are summarized in Table 1.

Table 1. Gene alterations in pancreatic cancers in humans and hamsters [34-60].

Gene	Alterations	Frequency in (%)	
		Human	Hamster (BOP-treated)
<i>K-ras</i>	Mutation	75–100	70–95
<i>p16^{INK4A}/CDKN2A</i>	CpG methylation/Deletion/Mutation	80–95	93
<i>DPC4/SMAD4</i>	Deletion / Mutation + LOH	50	8
<i>DCC</i>	Deletion	50	53
<i>P53</i>	Mutation + LOH	40–75	0
<i>FHIT</i>	Aberrant transcripts	62	73

LOH: loss of heterozygosity

K-ras is quite frequently mutated in pancreatic ductal carcinomas in hamsters (70–95%) [40-42] as well as humans (75–100%) [43-45], resulting in activation of downstream signaling proteins such as elements in the Raf/MEK/MAPK and PI3K/Akt pathways. *K-ras* mutations are also observed in early lesions, such as atypical ductal hyperplasia in hamsters and humans [41,46]. The major *K-ras* mutation in BOP-induced pancreatic carcinomas in hamsters is predominantly a G to A transition in the second position of codon 12, while both G to A transitions and G to T transversions at the second position of codon 12 are frequently observed in human pancreatic cancers [43,44].

The *p16^{INK4A}/CDKN2A* is known to be a tumor suppressor gene located at chromosome 9p that is inactivated in most pancreatic ductal carcinomas in humans (80–95%) by intragenic mutations (40%), homozygous deletions (40%) or hypermethylation of its promoter region (15%) [45,47,48]. The protein encoded by *p16* is an inhibitor of cyclin-dependent kinase and regulates the cell cycle by activation of RB proteins. Frequent alteration of *p16* (~93%) has also been reported in BOP-induced pancreatic tumors in hamsters and the majority of changes involve aberrant methylation (47%) or homozygous deletion (37%) [49].

DPC4/SMAD4 is a tumor suppressor gene located at chromosome 18q21.1 which encodes a protein associated with the TGF- β signaling pathway. *DPC4/SMAD4* is inactivated in 50% of pancreatic adenocarcinomas in humans by homozygous deletions (30%) or intragenic mutations in one allele coupled with loss of heterozygosity (LOH) (20%) [50]. On the other hand, *Dpc4/Smad4* alterations are rare in BOP-induced pancreatic tumors in hamsters (8%) [51].

DCC is a tumor suppressor gene located at chromosome 18q21.3, which encodes a protein with homology to cell adhesion receptors. Expression has been found to be lost in 50% of human pancreatic