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平成２３年度　総括・分担研究報告書

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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-Rbl, 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.

Obesity animal models including *ob/ob* mice, *db/db* mice and Zucker rats are well established,[3–5] and it has been reported that intraperiotoneal 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-Rbl, 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-Rbl 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, polydypsia, impaired glucose tolerance, hyperinsulinemia and hyperlipidemia[10] 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]

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
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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 DNA 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- CACGATTTCCCAGA GAACATGTG), leptin (5′ primer- CCAAAACCCTCATCAA GACC, 3′ primer- GTCCAACTGTTGAAGAATGTCCC), monocyte chemoattractant protein-1 (MCP-1) (5′ primer- CCACTCACCTGCTGCTACTCAT, 3′ primer- TGGTGATC CTCTTGTAGCTCTCC), Ob-Rbl (5′ primer- CCATCTTTTA 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× dilution. As the secondary antibody, biotinylated anti-goat IgG (Vector Laboratories, Burlingame, CA) was used at 200× 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× 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× 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 μg microsomes, 20 μL of 500 mM phosphate buffer (pH 7.4) and 20 μL of 500 μM chlorzoxazone solution were preincubated for 5 min at 37°C (total volume: 180 μL). Then, 20 μL of NADPH-generating system (100 mM $MgCl_2$, 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 μL 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 ± 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 ± 0.9 g (mean ± SD) and 17.4 ± 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 ± 2.7 g (mean ± SD) and 21.4 ± 1.5 g, and food intake were 4.0 ± 0.5 g/mouse/day and 3.0 ± 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 ± 4.9 g and 25.2 ± 1.5 g, and food intake were 5.2 ± 0.5 g/mouse/day and 3.6 ± 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 ± 106.1 mg/dL (mean ± SD), 101.6 ± 12.5 mg/dL, 1796 ± 493 μEq/L and 10.1 ± 3.9 μg/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 1*a* and 1*b*, 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 ± 1.1 g/mouse (at 13 weeks of age) and 11.3 ± 2.3 g/mouse (at 19 weeks of age).

Histopathological examination of visceral adipose tissue clearly showed enlargement of adipocytes (Figs. 1*c* and 1*d*), which was confirmed by quantification of the number of adipocyte nuclei observed in the field of fat tissue in KK-A^y mice (Fig. 1*e*). 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. 1*f*). 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 2*a* (13 weeks) and 2*c* (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. 2*b* and 2*d*). 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.

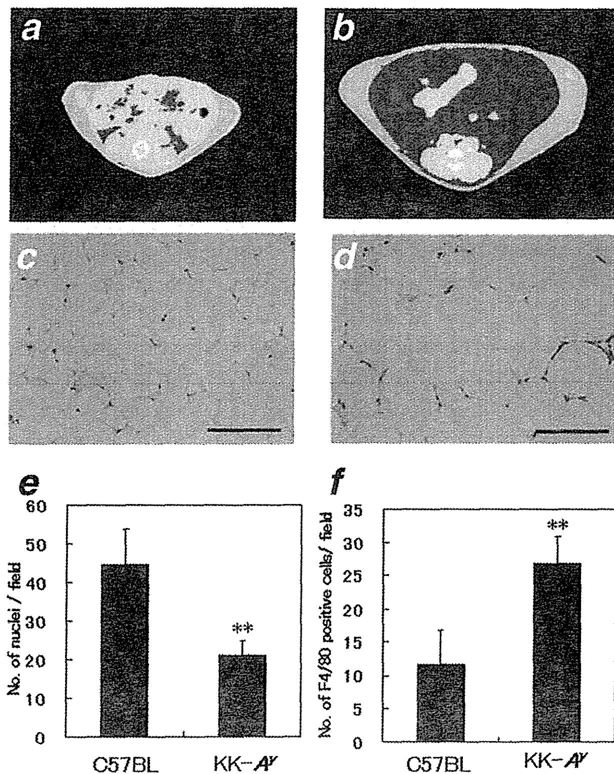

Figure 1. Obese features observed in KK-A^y mice at 13 weeks. Abdominal fat scanned by micro-CT was reconstructed to axial images (yellow represents subcutaneous fat and red represents visceral fat) for C57BL/6J (*a*) and KK-A^y mice (*b*). Histopathological sections of visceral fat in C57BL/6J (*c*) 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 ± SE. Bar represent 200 μm. **$p < 0.01$ *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 μg/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. 3*a*). 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

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

Data are mean ± 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).

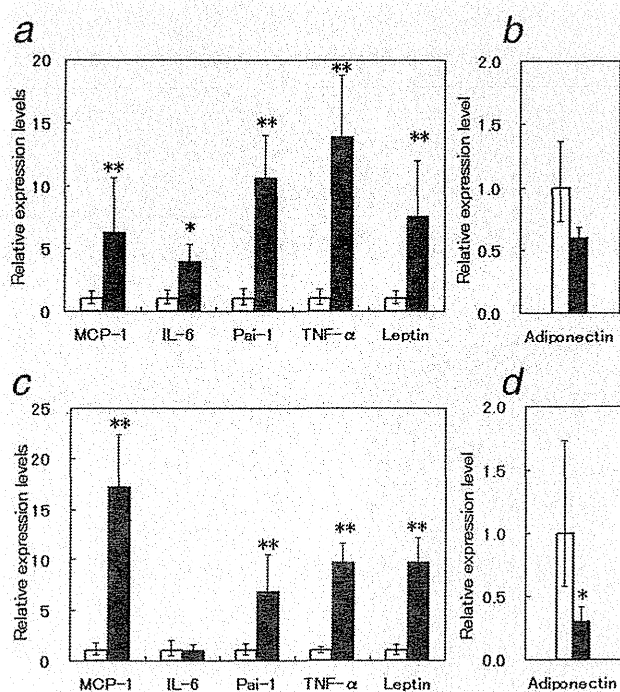

Figure 2. Relative expression levels of adipocytokine mRNA in visceral fat tissue of KK-A^y and C57BL/6J mice treated with AOM. RT-PCR analysis was performed to obtain MCP-1, IL-6, Pai-1, TNF-α, leptin and adiponectin mRNA expression levels at 13 weeks (*a* and *b*) and 19 weeks (*c* and *d*). GAPDH mRNA was used to normalize the data. KK-A^y mice (■); C57BL/6J mice (□). Data are means ± 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 μg/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					Mean no. of ACs/focus
		Proximal	Middle	Distal	Rectum	Total	
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-A^y	10/10	12.3 ± 11.4**	20.3 ± 9.0**	30.3 ± 2.3**	6.7 ± 1.0*	69.6 ± 12.9**	1.6 ± 0.2

Data are mean ± 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

		No. of mice with tumors (%)			No. of tumors/mouse		
Mice	**No. of mice**	**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-A^y	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 $^{**}p < 0.01$.

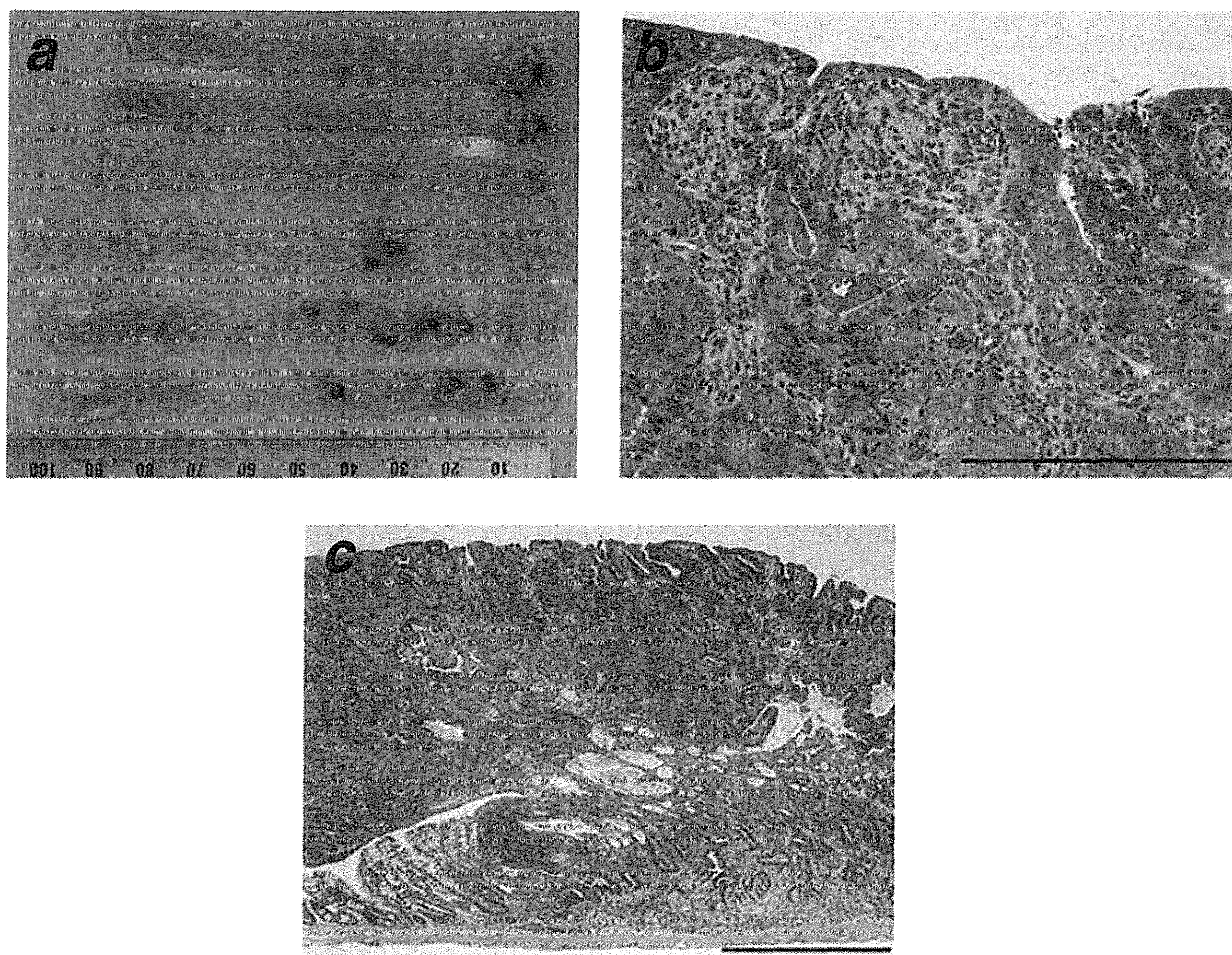

Figure 3. Macroscopic and histopathological analysis of colorectal tumors. (*a*) Representative macroscopic features of colorectal tumors developing in KK-A^y (lower three colorectal segments) and C57BL/6J mice (upper three colorectal segments) with a ruler. (*b*) Representative adenocarcinoma in KK-A^y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

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 vasolidation, 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-Rbl 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 adenocarcimnoma.[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.

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