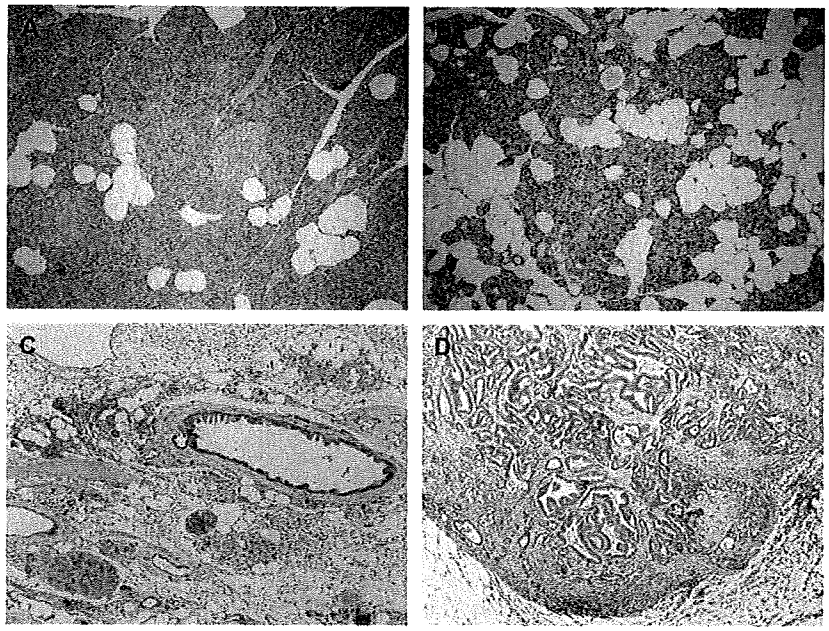

Fig. 2. Immunohistochemical staining for iNOS. No iNOS immunoreactivity is apparent in normal pancreas of non-treated (**A**) and BOP-treated (**B**) hamsters. Immunoreactive iNOS is observed in macrophages and islet cells in inflammation, atypical hyperplasia in ducts (**C**) and carcinoma epithelial cells (**D**) in BOP-treated hamsters. (**A–D**) ×100.

than in the saline + basal diet group ($P < 0.01$). However, in the BOP-treated groups, no significant differences were observed after treatment with ONO-1714.

Pancreatic lesions were histopathologically diagnosed as atypical hyperplasia, non-invasive adenocarcinomas and invasive adenocarcinomas. Incidence and multiplicity data are summarized in Table II. The incidence of atypical hyperplasia and adenocarcinomas induced by BOP was lower in the group treated with 200 p.p.m. ONO-1714 than in the control group (21 versus 52% at $P < 0.005$ and 45 versus 69% at $P < 0.05$, respectively). Remarkably, the incidence of invasive adenocarcinomas was significantly lower in the 200 p.p.m. ONO-1714 group than in the control group (12 versus 45% at $P < 0.001$). Multiplicities of total adenocarcinomas were significantly decreased by treatment with 100 p.p.m. (0.76 ± 0.82, $P < 0.05$) and 200 p.p.m. ONO-1714 (0.60 ± 0.77, $P < 0.01$) compared with the control value (1.19 ± 1.10). It was notable that the multiplicities of invasive adenocarcinomas in the 100 p.p.m. and 200 p.p.m. ONO-1714 groups were only one-half (0.36 ± 0.62, $P < 0.05$) and one-fifth (0.14 ± 0.42, $P < 0.01$) of the control value (0.74 ± 1.01), respectively. On the other hand, the multiplicities of non-invasive adenocarcinomas did not significantly differ among the three groups. Figure 3 shows the size distribution of pancreatic adenocarcinomas. The numbers of carcinomas <3, 3–5 and ≥5 mm in diameter in the BOP + 100 p.p.m. ONO-1714 group were 55, 56 and 86% of the BOP + basal diet values, respectively, suggesting that treatment with 100 p.p.m. ONO-1714 tended to suppress the development of carcinomas <5 mm in diameter, but not the larger lesions. On the other hand, those in the BOP + 200 p.p.m. ONO-1714 group were 75, 38 and 29% of the control group, respectively, indicating that treatment with 200 p.p.m. ONO-1714 tended to suppress the development of carcinoma >3 mm in diameter and significantly reduced the development of carcinoma >5 mm in diameter ($P < 0.05$).

In addition to pancreatic ductal tumors, tumors of the bile duct, liver, lungs and kidneys have been reported to be induced by BOP in hamsters (18). In the present study, hepatocellular and cholangiocellular tumors were observed in the BOP-treated group at incidences of 12 and 50%, respectively (Table III). The cholangiocellular tumors developed in both intra- and extrahepatic bile ducts. The incidences of hepatocellular and cholangiocellular tumors were not significantly changed by ONO-1714 administration, but the multiplicity of cholangiocellular tumors was significantly decreased by 200 p.p.m. ONO-1714 treatment compared with the controls (0.38 ± 0.66 versus 1.14 ± 1.57 at $P < 0.005$) (Table III).

Table I. Final body weights of the hamsters and average food intake

Treatment	No. of animals	Final body weight (g)	Food intake[a] (g/hamster/day)
BOP + basal diet	42	199 ± 21[b,c]	10.8 ± 0.4[c]
BOP + 100 p.p.m. ONO-1714	42	200 ± 17	11.0 ± 0.6
BOP + 200 p.p.m. ONO-1714	42	193 ± 19	10.5 ± 0.5
Saline + basal diet	6	219 ± 18	11.7 ± 1.0
Saline + 100 p.p.m. ONO-1714	6	194 ± 22	10.8 ± 0.1
Saline + 200 p.p.m. ONO-1714	6	190 ± 13[d]	10.1 ± 0.1

[a]Total food intake of each animal cage for 15 weeks was divided by animal number in each cage and the total period (days).
[b]Data are mean ± SD.
[c]Significantly different from the saline + basal diet group at $P < 0.05$.
[d]Significantly different from the saline + basal diet group at $P < 0.01$.

In contrast, the incidences and multiplicities of lung tumors were statistically increased by 100 p.p.m. ONO-1714 [33/42 (79%) at $P < 0.05$ and 1.60 ± 1.29 at $P < 0.05$, respectively] and slightly but not significantly by 200 p.p.m. ONO-1714 [26/42 (62%) and 1.26 ± 1.33, respectively] compared with the control group [21/42 (50%) and 0.98 ± 1.42, respectively]. A renal mesenchymal tumor and a hemangioma were observed in the BOP + basal diet group, an angiosarcoma in the BOP + 100 p.p.m. ONO-1714 group and a nephroblastoma in the BOP + 200 p.p.m. ONO-1714 group, but their incidences were not significant. Tumors in the pancreatic duct, bile duct, liver, lungs and kidneys were not observed in the saline vehicle ($n = 15$) or 100 p.p.m. and 200 p.p.m. ONO-1714 group hamsters without the BOP treatment ($n = 15$, each).

Table II. Effects of ONO–1714 treatment on the incidences and multiplicities of pancreatic lesions induced by BOP[a]

Dose of ONO-1714 in diet	Effective no. of animals	No. of animals with lesions (%)				No. of lesions in the pancreas			
		Atypical hyperplasia	Ductal adenocarcinoma			Atypical hyperplasia	Ductal adenocarcinoma		
			Non-invasive	Invasive	Total[b]		Non-invasive	Invasive	Total[b]
0 p.p.m.	42	22 (52)[c]	15 (36)	19 (45)	29 (69)	0.57 ± 0.59[d]	0.45 ± 0.71	0.74 ± 1.01	1.19 ± 1.10
100 p.p.m.	42	24 (57)	13 (31)	12 (29)	24 (57)	0.69 ± 0.68	0.40 ± 0.70	0.36 ± 0.62*	0.76 ± 0.82*
200 p.p.m.	42	9 (21)**	15 (36)	5 (12)***	19 (45)*	0.29 ± 0.64	0.45 ± 0.71	0.14 ± 0.42***	0.60 ± 0.77**

[a]Hamsters were fed a basal diet or a diet containing ONO-1714, an iNOS inhibitor, for 15 weeks.
[b]The total represents animals with non-invasive and/or invasive carcinomas.
[c]Percentages in parentheses.
[d]Data are mean ± SD values.
Significantly different from the control group at $^*P < 0.05$, $^{**}P < 0.005$ and $^{***}P < 0.001$.

Discussion

The present study demonstrated that iNOS is expressed in pancreatic cancer cells and that the iNOS inhibitor, ONO-1714, can effectively suppress the development of atypical hyperplasia and cancer, especially invasive cancers, in the hamster pancreas after treatment with BOP. The results indicated that iNOS plays important roles in the development of preneoplastic lesions at an early stage of pancreatic carcinogenesis and also in cancer invasion and expansion in later stages.

In our previous study of colon carcinogenesis, ONO-1714 suppressed the development of rat colon tumors >3 mm in diameter (16), in line with the present findings. It has been reported that angiogenesis is necessary to supply oxygen and nutrients to solid tumors >1–2 mm^3 in size (28). NO enhances vascular permeability, partly through activation of matrix metalloproteinases (29), suggesting that suppression of the development of large tumors in ONO-1714-treated groups may be associated with inhibition of angiogenesis by the iNOS inhibitor.

Expression of iNOS has been detected in more than half of human pancreatic cancers (7–9). Here, iNOS expression was observed in most of the hamster pancreatic cancers and atypical hyperplasia. In the BOP-induced pancreatic ductal carcinogenesis model in hamsters, G to A transitions at the second base of the codon 12 of the K-*ras* gene have been shown to be quite frequent in pancreatic cancers and even in preneoplastic lesions at lower frequency (30). Our previous study revealed that iNOS expression can be markedly elevated by transfection of K-*ras* mutant cDNA into IEC-6 rat intestinal epithelial cells in the presence of IL-1β or lipopolysaccharide through the activation of promoters on nuclear factor κB, C/EBP and CRE-like sites and that growth of tumors formed in nude mice by subcutaneous injection of the K-*ras* mutant-transfected cells can be suppressed by feeding diets containing NOS inhibitors (15). It is feasible that iNOS expression in pancreatic cancers could also be associated with K-ras activation. Indeed, human pancreatic cancers frequently harbor K-*ras* mutations (21,31–33) and other cancers with frequent K-*ras* mutations, such as colon (32,33), lung (33) and intrahepatic bile duct carcinomas (34), also show increased iNOS expression (35–37). Thus, NO produced by iNOS may be generally involved in tumor development by activated K-ras, and iNOS-selective inhibitors should be considered as possible candidates for the prevention of all cancers featuring K-ras activation.

K-*ras* mutations are observed from early stages of carcinogenesis in the pancreas, colon, lungs (30,33) and intrahepatic bile ducts (38). Our previous studies in the azoxymethane-induced rat colon carcinogenesis model showed frequent K-*ras*-activating mutations in hyperplastic aberrant crypt foci (39) and suppression of aberrant crypt focus development by the iNOS inhibitor ONO-1714 (16). The present study also showed iNOS expression in precancerous lesions and suppression of the development of atypical hyperplasia in the pancreas of hamsters by an iNOS inhibitor. It can thus be concluded that

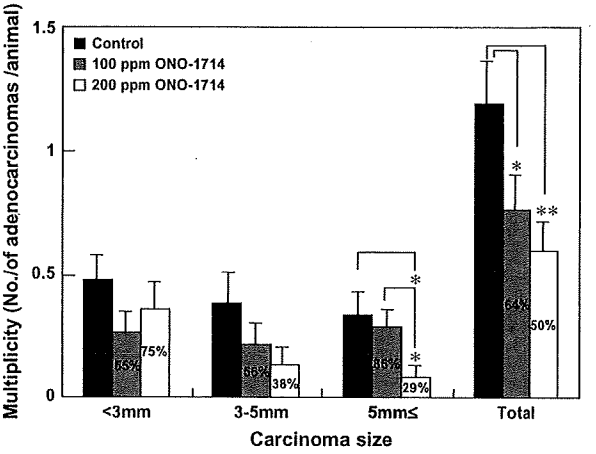

Fig. 3. Effects of ONO-1714 treatment on the sizes of pancreatic cancers. The numbers of each size of pancreatic cancers per hamster are given for the BOP + 0 p.p.m. ONO-1714 (black bars), BOP + 100 p.p.m. ONO-1714 (gray bars) and BOP + 200 p.p.m. ONO-1714 (white bars) groups (mean ± standard error values). * and ** Significantly different from the respective controls at $P < 0.05$ and $P < 0.01$, respectively. The percentage of the BOP + basal diet control value is shown in each column.

K-ras-enhanced iNOS expression may contribute to the development of early precancerous lesions.

It has been reported that IL-1β induces iNOS expression in pancreatic β-cells (40), and overproduction of NO causes dysfunction and destruction of β-cells (41). In the present study, iNOS expression in pancreatic islets surrounded by cancer-associated inflammation was observed. Epidemiological studies have reported that diabetes mellitus is also a risk factor for pancreatic cancer (3). Therefore, increased expression of iNOS in pancreatic islets may also be involved in pancreatic carcinogenesis and iNOS inhibitors might also be protective against autoimmune diabetes.

In the present study, the iNOS inhibitor ONO-1714 significantly suppressed the development of pancreatic cancer and cholangiocellular tumors, but not of lung tumors. Pancreatic ductal adenocarcinomas and cholangiocellular tumors in BOP-treated hamsters have certain genetic characteristics in common; for example, K-*ras* mutations and aberrant transcription of the *fragile histidine triad* gene were observed in both (19,20,38,42). Expression of iNOS was also observed in cholangiocellular tumors obtained in the present study (data not shown). It could be speculated that the influence of the iNOS inhibitor might be similar for the two types of carcinoma, one arising from ducts in the pancreas and the other from bile ducts. In contrast, treatment with 100 p.p.m. but not 200 p.p.m. ONO-1714 rather enhanced development of

Table III. Effects of ONO–1714 treatment on the incidences and multiplicities of liver and bile duct tumors induced by BOP[a]

Dose of ONO-1714 in diet	Effective no. of animals	No. of animals with tumors (%)				No. of tumors in the liver			
		Hepatocellular adenoma	Cholangiocellular tumors			Hepatocellular adenoma	Cholangiocellular tumors		
			Adenoma	Adenocarcinoma	Total[b]		Adenoma	Adenocarcinoma	Total[b]
0 p.p.m.	42	5 (12)[c]	19 (45)	4 (10)	21 (50)	0.12 ± 0.33[d]	1.02 ± 1.52	0.12 ± 0.40	1.14 ± 1.57
100 p.p.m.	42	2 (5)	23 (55)	2 (5)	24 (57)	0.05 ± 0.22	0.76 ± 0.88	0.07 ± 0.34	0.83 ± 0.91
200 p.p.m.	42	3 (7)	12 (29)	3 (7)	13 (31)	0.07 ± 0.26	0.31 ± 0.52*	0.07 ± 0.26	0.38 ± 0.66**

[a]Hamsters were fed a basal diet or a diet containing ONO-1714, an iNOS inhibitor, for 15 weeks.
[b]The total represents animals with non-invasive and/or invasive carcinomas.
[c]Percentages in parentheses.
[d]Data are mean ± SD values.
Significantly different from the control group at $^{*}P < 0.01$ and $^{**}P < 0.005$.

lung tumors. It has been reported that several NOS inhibitors are chemopreventive in the rat tracheal epithelial cell transformation system (43). Reduction in lung tumor development in iNOS(−/−) mice has also been reported (44), suggesting that iNOS expression is associated with lung tumorigenesis. However, endothelial NOS and neuronal NOS are also expressed in lung tumors and high total expression levels of the three NOS types have been suggested to be a favorable prognostic sign (45). Thus, it appears that data on roles of NO in lung tumorigenesis are contradictory and the promotive effect of iNOS is not yet conclusive. Interestingly, other chemopreventive agents reported, such as 4-phenylbutyl isothiocyanate (46), phenethyl isothiocyanate (47) and a cyclooxygenase inhibitor, nimesulide (48) suppressed pancreatic cancers and lung tumors, but enhanced (46) or did not affect (47,48) liver tumorigenesis in BOP-treated hamsters. Thus, it can be presumed that the inhibitory mechanisms of these agents on pancreatic cancer may be different from that of ONO-1714. Our previous study on the prevention of hamster pancreatic carcinogenesis by a peroxisome proliferator-activated receptor γ ligand, pioglitazone, also showed significant suppression of the development of pancreatic ductal adenocarcinomas and cholangiocellular tumors, but not of lung adenomas (26). It is known that peroxisome proliferator-activated receptor γ activation inhibits cytokine-mediated iNOS expression (49,50), indicating that inhibitory mechanisms of ONO-1714 and pioglitazone on hamster pancreatic carcinogenesis could be shared, at least in part.

In conclusion, the present study demonstrated probable involvement of iNOS expression in hamster pancreatic ductal carcinogenesis, and suppression of development of pancreatic atypical hyperplasia and invasive adenocarcinomas by treatment with an iNOS inhibitor. Thus, it is proposed that iNOS inhibitors might be promising chemopreventive agents against pancreatic cancer.

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Overexpression of low-density lipoprotein receptor and lipid accumulation in intestinal polyps in Min mice

Michihiro Mutoh*, Masami Komiya, Naoya Teraoka, Toshiya Ueno, Mami Takahashi, Tsukasa Kitahashi, Takashi Sugimura and Keiji Wakabayashi

Cancer Prevention Basic Research Project, National Cancer Center Research Institute, Chuo-ku, Tokyo, Japan

***Apc*-deficient Min mice feature low expression of lipoprotein lipase (LPL), high concentration of serum triglyceride (TG), fatty change of the liver and large numbers of intestinal polyps. We have reported that induction of LPL expression reduces serum lipid, especially TG, improves fatty change of the liver and inhibits intestinal polyp formation in the mice. In this study, fatty change/lipid accumulation in intestinal mucosa and polyps in Min mice were analyzed by Oil-red O staining and electron microscopy. A number of large lipid droplets were found in the epithelia of the upper part of polyps. On the other hand, small lipid droplets were only slightly observed at the tip of the villi in non-tumoros parts of the small intestine of Min mice and in the villi of wild-type mice. Moreover, low-density lipoprotein receptor (LDLR) was overexpressed in the area where lipid droplets were observed. The expression levels of LDLR mRNA in the intestinal polyps of Min mice were ~3 times higher compared to those in the non-tumoros parts. Remarkable expression of cyclooxygenase-2 was mainly distributed in stromal cells and some in epithelial cells. It is speculated that lipid accumulation in the intestinal polyps may play an important role in intestinal polyp formation in *Apc*-deficient mice.**

Key words: *Apc*-deficient mice; low-density lipoprotein receptor; lipid accumulation; triglyceride; intestinal polyp

The incidence and mortality of colon cancer, one of the most common cancers, is increasing in developed countries. High fat diet consumption, obesity and hyperlipidemia, especially hypertriglyceridemia, are linked to the increased risk of colorectal tumor.[1–5] Recently, we reported an age-dependent hyperlipidemic state in *adenomatous polyposis coli* (*Apc*)-deficient Min and *Apc*^{1309} mice, animal models of familial adenomatous polyposis (FAP).[6,7] *Apc*-deficient mice develop large numbers of intestinal polyps due to a truncation mutation in the *Apc* gene leading to an activation of Wnt signaling to promote cell growth.

Although the direct link between *Apc*-deficiency and hyperlipidemia has yet to be clarified, it is notable that serum triglyceride (TG) levels in *Apc*-deficient mice are almost 10-fold more than those observed in wild-type littermates (C57BL/6J).[6] TG is rich in lipoproteins such as chylomicrons (CM), very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL), and is hydrolyzed by lipoprotein lipase (LPL) to free fatty acids and monoacylglycerol. High populations of lipoproteins are VLDL (~50%) and LDL (~25%), TG-rich lipoproteins, in Min mice, while high-density lipoprotein (~80%) in wild-type mice.[6–8] Physiologically, decrease or deficiency of LPL is associated with hyperlipidemic state[9,10] and *Apc*-deficient mice showed decreased expression levels for LPL mRNA in the liver and small intestine, supposed to be the reason for hypertriglyceridemic status. In addition to high concentration of the lipid in the serum, steatosis of the liver is observed in Min mice, which was confirmed by staining frozen sections with Oil-red O.[6] Moreover, we have demonstrated that induction of LPL mRNA by peroxisome proliferator-activated receptor (PPAR)-α and -γ agonists and a 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] However, fatty changes in the intestinal mucosa including lipid accumulation in Min mice intestinal polyp have remained to be elucidated.

Regarding intestinal digestion of dietary fat, fat in the intestinal content changes to micellar solubilization form due to the action of amphipathic bile salts and pancreatic lipase.[11] Further hydrolysis by pancreatic lipase to form smaller micelles (30–100 Å) helps lipid absorption through small intestinal epithelia, and this lipid absorption would occur through passive diffusion and through endocytosis-dependent pathways using receptors. The LDL receptor (LDLR) has been reported to regulate the majority of essential fatty acid uptake as well as cholesterol uptake into cells.[12] Moreover, it has been reported that LDL plays an important role in cell growth of human colon cancer cells.[13] After penetration of micelles, through the brush border of the small intestine, CM are synthesized in the epithelial cells. Then, CM is released from small intestinal epithelial cells to circulate through the lymphatic system and finally, taken up by the liver.

It is possible that the efficiency of intestinal lipid absorption is increased in the *Apc*-deficient mice and induces lipid accumulation in the intestinal mucosa. In this study, we examined the lipid accumulation in intestinal polyps developed in Min mice by Oil-red O staining and electron microscopy examination and found a number of large lipid droplets accumulated in the epithelium of polyps. Moreover, immunohistochemical staining revealed that LDLR and cyclooxygenase-2 (COX-2) were overexpressed in the epithelia where lipid droplets were observed. The possible mechanisms inducing lipid accumulation in intestinal polyps and the effects of lipid accumulation on the development of polyps in *Apc*-deficient mice are also discussed.

Material and methods

Animals

Male C57BL/6-*Apc*$^{Min/+}$ mice (Min mice) were purchased from The Jackson Laboratory at 6 weeks of age and genotyped as previously reported.[14] Heterozygotes of the Min strain and wild-type (C57BL/6J) mice were acclimated to laboratory conditions for 1 week. Five mice were housed per plastic cage with sterilized softwood chips as bedding in a barrier-sustained animal room at 24°C ± 2°C and 55% humidity on a 12 hr light/dark cycle.

To investigate the lipid accumulation in intestinal polyps, male Min mice (n = 5) and wild-type mice (n = 5) at 7 weeks of age were given AIN-76A powdered basal diet (CLEA Japan, Tokyo, Japan) for 8 weeks. Food and water were available *ad libitum*. The animals were observed daily for clinical signs and mortality. Body weights and food consumption were measured weekly. 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.

Abbreviations: CM, chylomicrons; COX-2, cyclooxygenase-2; FAP, familial adenomatous polyposis; LDL, low-density lipoprotein; LPL, lipoprotein lipase; PPAR, peroxisome proliferator-activated receptor; TG, triglyceride; VLDL, very low-density lipoprotein.

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***Correspondence to:** Cancer Prevention Basic Research Project, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. Fax: +81-3-3542-9305. E-mail: mimutoh@ncc.go.jp

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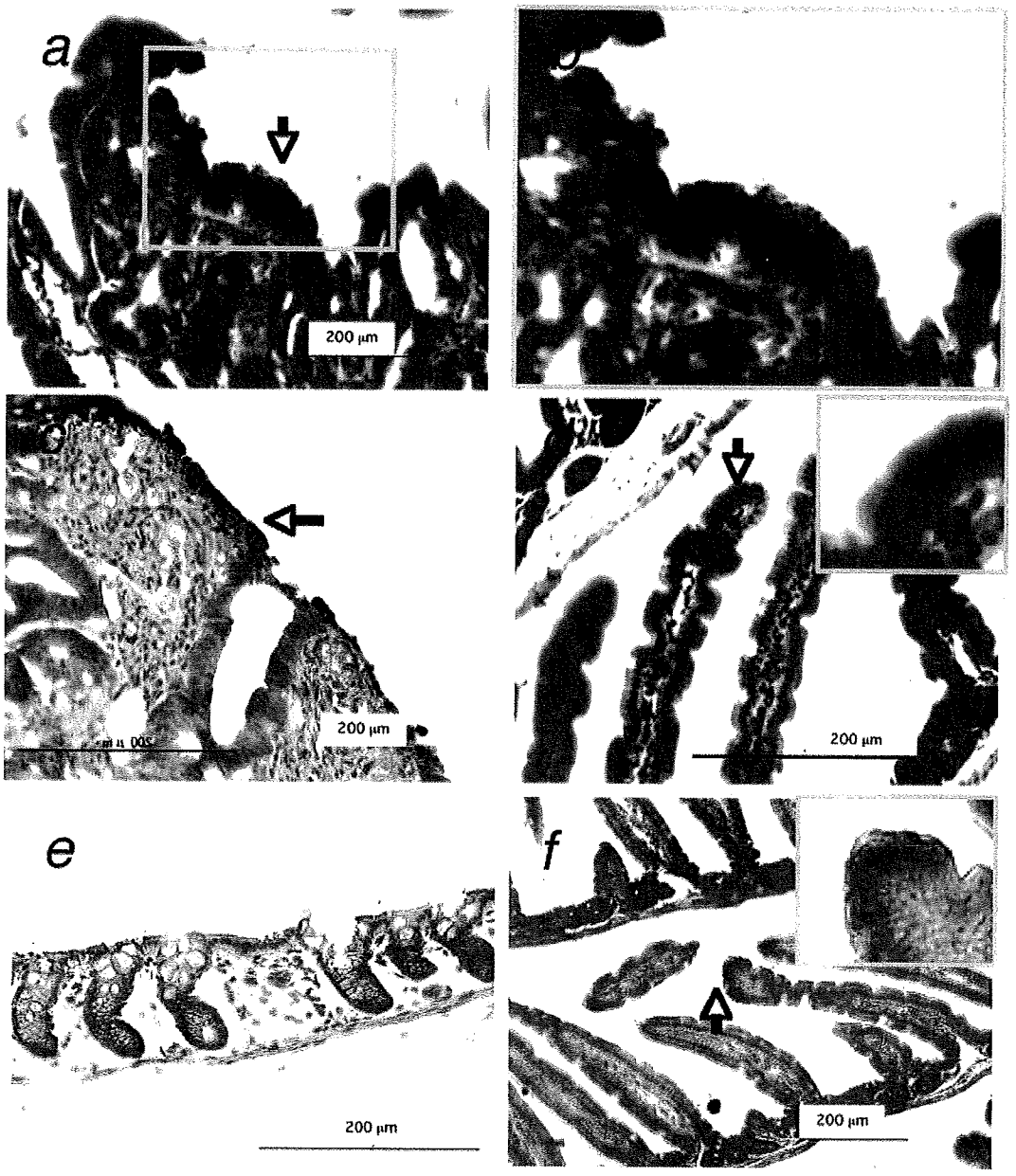

FIGURE 1 – Lipid accumulation observed in epithelial cells of polyp part of Min mice. Frozen sections of intestinal tissues were stained with Oil-red O as described in the text. Lipid droplets were stained in red. Arrows indicate the lipid droplets. Bars represent 200 μm. (*a*) Small intestinal polyp part of Min mice. (*b*) Large magnification of lipid droplets shown in yellow square part of (*a*). (*c*) Colon polyp part of Min mice. (*d*) Non-tumorous part of small intestinal tissue of Min mice. Larger magnification of a pointed part was inserted with the square. (*e*) Non-tumorous part of colon tissue of Min mice. (*f*) Small intestinal tissue of wild-type mice. Larger magnification of a pointed part was inserted with the square.

Intestinal polyp assessment

The intestinal tract was removed and separated into the small intestine, cecum and colon. The small intestine was divided into the proximal segment (4 cm in length), and then the middle and distal halves of the remainder. All polyps in the proximal segments were picked up under a stereoscopic microscope and the remaining intestinal mucosa was removed by scraping, and then both stored at −80°C. The stored samples from proximal segments were used for further RT-PCR analysis. Other segments ($n = 5$) were opened longitudinally and fixed flat between sheets of filter paper in 10% buffered formalin[6] for further Oil-red O staining and immunohistochemical staining.

Oil-red O staining

Fat droplets within digestive tissues, such as esophagus, glandular stomach, small intestine and colon, were observed using a

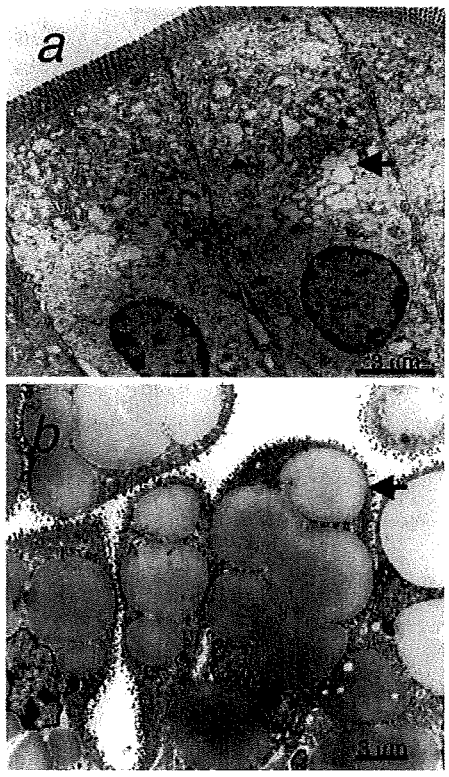

FIGURE 2 – Transmission electron microscopy of epithelial cells of non-tumorous part and polyp part of the small intestine in Min mice. Bars represent 3 μm. (*a*) Non-tumorous part of the small intestine in Min mice. Magnification ×3,500. (*b*) Small intestinal polyp part of Min mice. Arrows indicates one of the lipid droplets. Magnification ×1,800.

modified Oil-red O staining method.[15] In brief, frozen sections were treated with 60% 2-propanol for 1 min, stained with Oil-red solution for 15 min at room temperature. The dye was then removed, and the sections were washed with PBS and 60% 2-propanol each for 2 min. Images of tissues stained with Oil-red O were obtained with an Olympus digital camera.

Immunohistochemical staining

The middle and distal segments of the small intestines were fixed, embedded and sectioned as Swiss rolls for further immunohistochemical examination with the avidin-biotin complex immunoperoxidase technique, and polyclonal goat anti-mLDLR antibody (R&D systems, Minneapolis, MN) at 50× dilution, polyclonal rabit anti-p-Akt antibody (Ser 473; Santa Cruz Biotechnology, Santa Cruz, CA) and polyclonal goat anti-COX-2 antibody (C-20; Santa Cruz Biotechnology) at 100× dilution. As the secondary antibody, biotinylated anti-goat IgG, and absorbed with rat serum (Vector Laboratories, Burlingame, CA) was employed at 200× dilution. Staining was performed using avidin-biotin reagents (Vectastain ABC reagents; Vector Laboratories), 3,3′-diaminobenzidine and hydrogen peroxide, and the sections were counterstained with hematoxylin to facilitate orientation. As a negative control, consecutive sections were immunostained without exposure to the primary antibody.

Western blot analysis

Samples from small intestinal polyps and non-tumorous parts of small intestines in Min mice were lysed in 100 μl lysis buffer [0.0625 M Tris-HCl (pH 6.8), 20% 2-mercaptoethanol, 10% glycerol, 5% SDS]. Thirty μg of protein were separated in 10% PAGE-SDS gels and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). Antibodies against the LDLR (R&D systems) and β-actin (Biomedical Technologies, Stoughton, MA) were used at a 1:1,000 dilution. Peroxidase-conjugated secondary antibodies for anti-rabbit IgG were obtained from GE Healthcare, UK. Blots were developed with ECL western blotting detection reagents (GE Healthcare, Buckinghamshire, UK).

Electron microscope

For transmission electron microscopy, tissues from middle segments of small intestine were fixed with 2.5% glutaraldehyde for 2 hr at 4°C. Specimens were fixed with 1% osmium tetroxide for an additional 2 hr at 4°C, dehydrated in alcohol and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate, and photographed using H-7500 transmission electron microscope (Hitachi High-Technologies, Japan).

Real-time PCR analysis

Tissue samples including polyp part and non-tumorous part from the proximal segments of mice were rapidly deep-frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated from tissues by using Isogen (Nippon Gene, Tokyo, Japan), treated with DNase (Invitrogen, Carlsbad, CA) and 3 μg aliquots in a final volume of 20 μl were used for synthesis of cDNA using an Omniscript RT Kit (Qiagen, Hilden, Germany) and an oligo(dT) primer. Real-time PCR was carried out using a DNA Engine Opticon TM 2 (MJ Japan, Tokyo, Japan) with SYBR Green Realtime PCR Master Mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Primers for mouse LDLR (5′primer-TCCAATCAATTCAGCTGTGG, 3′primer-GAGCCATCTAGGC AATCTCG) and GAPDH (5′primer-TTGTCTCCTGCGAC TTCA, 3′primer-CACCACCCTGTTGCTGTA) were employed.[16] To assess the specificity of each primer set, amplicons generated from the PCR reaction were analyzed for melting curves and also by electrophoresis in 2% agarose gels.

Statistical analysis

The student's *t*-test was used for statistical analyses of the intestinal LDLR mRNA level. Differences were considered to be statistically significant at $p < 0.05$.

Results

Accumulation of lipids in intestinal polyps of Min mice

The body weights of Min mice and wild-type mice were almost the same during the study, and the final body weights were 26.3 and 28.7 g, respectively. Average daily food intake of Min mice and wild-type mice did not differ significantly. Consistent to the previous study, Min mice developed almost all polyps in the small intestine, with only a few found in the colon.[6] The average number of intestinal polyps developed in Min mice was 70.6 ± 14.8 (mean ± SD). However, none of the wild-type mice developed intestinal polyps.

To determine whether lipid accumulation was present in intestinal polyps and/or non-tumorous part, frozen sections of Swiss-rolled middle and distal parts of the small intestine were stained with Oil Red O solution and were examined. A number of large lipid droplets were found in the epithelium of upper part of polyps in the small intestine (Figs. 1*a* and 1*b*). In 2 of 3 colon polyps, a few large lipid droplets were also found in the epithelium of upper part of polyps (Fig. 1*c*). On the other hand, small lipid droplets were slightly observed at the tip of villi of non-tumorous parts in the middle and distal parts of small intestine of Min mice (Fig. 1*d*). There were no lipid droplets found in mucosa of non-tumorous part of colon (Fig. 1*e*). In the middle and distal parts of small intestinal mucosa of wild-type mice, small lipid droplets were slightly observed at the tip of the villi (Fig. 1*f*). High magnification at the bottom of villi showed small lipid droplets under the epithe-

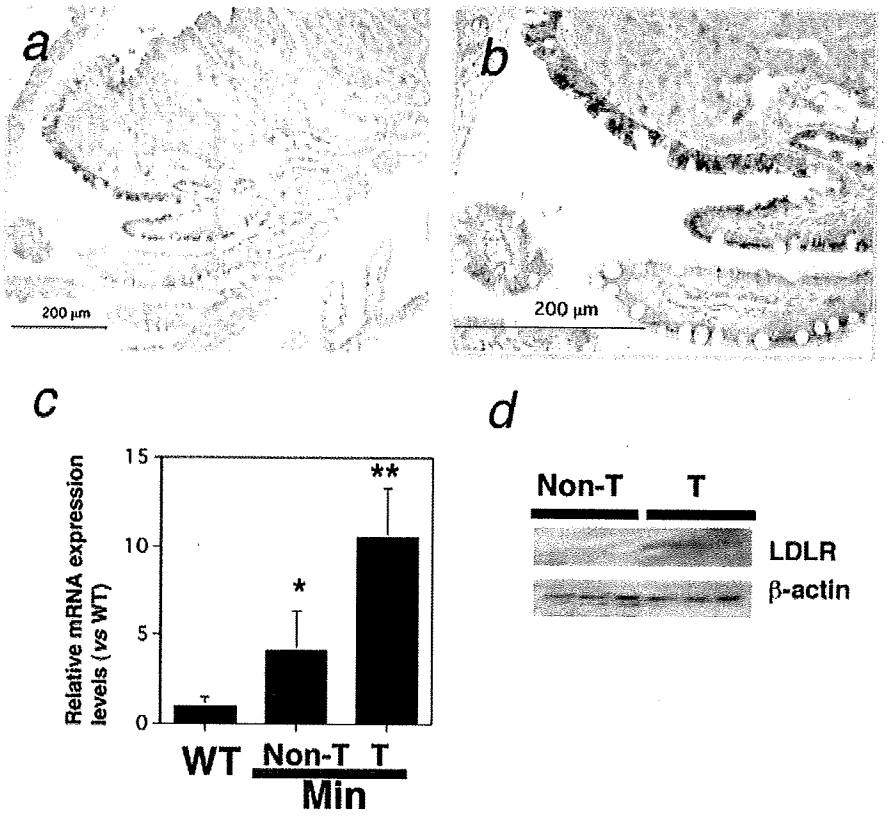

FIGURE 3 – LDLR overexpression in intestinal polyps. (*a*) Immunohistochemical staining of LDLR protein in polyp parts of the small intestine of a Min mouse. Bars represent 200 μm. (*b*) Large magnification of immunohistochemical staining of LDLR protein shown in yellow square part of (*a*). (*c*) Quantitative real-time PCR analysis of LDLR mRNA expression in intestinal mucosa of wild-type mice ($n = 5$), non-tumorous part of intestinal mucosa of Min mice and intestinal polyps of Min mice ($n = 5$). Data are means ± SD. Normalized by GAPDH. $*p < 0.05$ (*vs.* WT); $**p < 0.01$ (*vs.* WT or *vs.* Non-T). WT, small intestinal mucosa in wild-type mice; Non-T, small intestinal mucosa in non-tumorous part; T, tumorous part. (*d*) Western blot analysis of LDLR protein expression in intestinal mucosa of wild-type mice ($n = 3$), non-tumorous parts of intestinal mucosa of Min mice and intestinal polyps of Min mice ($n = 3$). β-actin was used as loading control.

lial cells, suggesting CM released from epithelial cells to circulate through the lymphatic system. Oil-red O staining was further performed in esophagus and glandular stomach, and revealed that there were no lipid droplets in mucosa of these organs (data not shown).

Examining under an electron microscope, microvilli, mitochondria and nuclei of epithelial cells from non-tumorous part of Min mice appeared intact, and small lipid droplets were observed in the cytoplasm (Fig. 2*a*). On the other hand, epithelial cells from intestinal polyps were shown to have huge lipid droplets, occupying almost all the cytoplasm with depressed nuclei (Fig. 2*b*).

Overexpression of LDLR in intestinal polyps of Min mice

We investigated the localization and expression levels of LDLR in polyps and non-tumorous part of Min mice. Strong immunohistochemical staining for LDLR was observed in epithelial cells at the surface of small intestinal polyps of Min mice, especially, in the area where lipid droplets were observed (Figs. 3*a* and 3*b*). Weak LDLR staining was observed in the non-tumorous part as shown in Figure 3*a*. These LDLR staining were not observed in the small intestinal villi of wild-type mice, and no artificial staining was detected in controls using the secondary antibody only (data not shown).

Real-time PCR revealed that LDLR mRNA level in intestinal polyps was obviously increased (~4 times) as compared with non-tumorous part of intestine in Min mice (Fig. 3*c*). LDLR mRNA levels in the Min mice polyps were also almost 10-fold elevated as compared with intestinal mucosa of wild-type mice (Fig. 3*c*). Moreover, a Western blot analysis was performed for protein expression levels of LDLR in intestinal tumoros parts and non-tumoros parts of normal mucosa, and weak but confidential expression levels of LDLR in intestinal tumor part were detected (Fig. 3*d*).

Localization of COX-2 in intestinal polyps of Min mice

To further explore possible mechanisms whereby an accumulated lipid leads to the epithelial cell proliferation and intestinal tumor promotion, localization of COX-2 in small intestinal polyps of Min mice was investigated. Consistent with previous reports,[17] levels of COX-2 protein were found to be very low in the normal mucosa, while the protein was found to be expressed in large quantities in the polyp stroma near the luminal surface (Fig. 4*a*). Interestingly, in some part, COX-2 staining was observed in the cytoplasm of epithelial cells (Fig. 4*b*). As demonstrated in Figure 4*c*, Akt phosphorylation was observed ubiquitously in the epithelial cells of intestinal polyps.

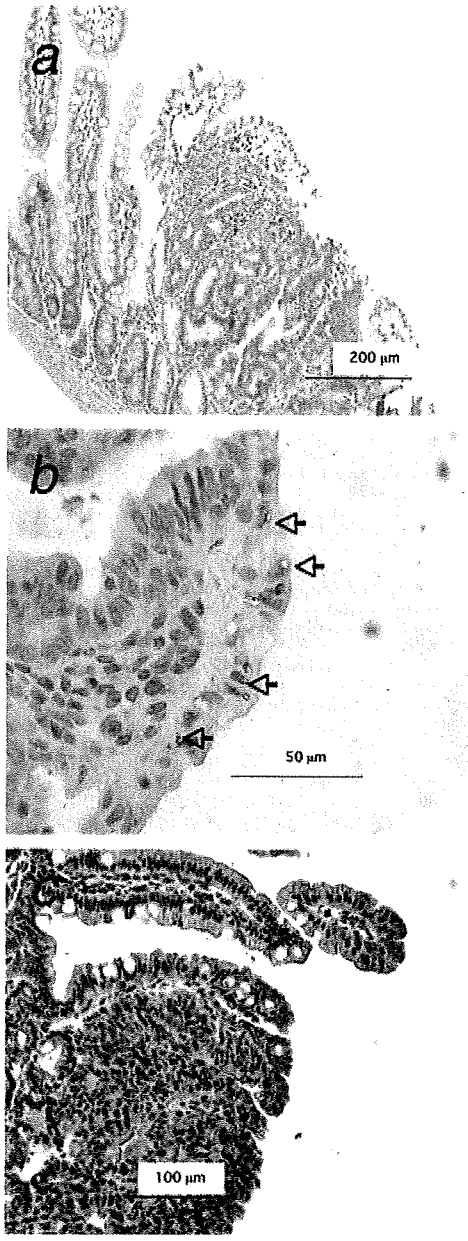

FIGURE 4 – Immunohistochemical staining of COX-2 protein in intestinal polyps of Min mice. (*a*) COX-2 expression in stromal cells of the small intestinal polyps. Bars represent 200 μm. (*b*) COX-2 expression shown in epithelial cells of Min mouse intestinal polyp. Arrows indicate COX-2 staining. Bars represent 50 μm. (*c*) Phosphorylated Akt expression shown in epithelial and stromal cells of Min mouse intestinal polyp. Bars represent 100 μm.

Discussion

In this study, lipid droplets were observed in the small intestinal and colorectal polyps of Min mice, occurring at the surface epithelium and coinciding with an overexpression of LDLR found in the same area. COX-2 was also expressed in stromal and epithelial cells. It is therefore speculated that lipid accumulation in the intestinal polyps may play an important role in intestinal polyp development in *Apc*-deficient mice.

To our knowledge, lipid accumulation in the tumor epithelial cells has not yet been reported in experimental colon carcinogenesis models, although that in human colon cancer was reported quite recently.[18] It has been observed that number of lipid droplets increased in human colon adenocarcinoma cells when compared with an adjacent normal cells from the same patient submitted to surgical resection. In this study, many large lipid droplets were found in the epithelium of upper part of polyps, while small lipid droplets were only slightly observed at the tip of the villi in non-tumorous parts of Min mice. Dietary fat moves in intestinal tract, contacting with the tip of the villi. Lipid may be largely absorbed at the tip of the villi. Moreover, accumulation of lipids is localized in the cytoplasm of intestinal epithelial cells, but not in stromal cells, supporting the idea that the origin of accumulated lipids are from the content in digestive tract and not from blood vessels.

From our results, it is speculated that there are 2 possible mechanisms regarding intestinal tumor cell growth. One might be the direct effect of TG or LDL receptor-mediated stimuli on epithelial cells, and the other might be the effect of prostaglandin E_2 (PGE_2) produced by COX-2 in stromal cells on epithelial cells. Recent evidence indicates that the members of the LDL receptor gene family could activate the phosphoinositide (PI) 3-kinase-mediated pathway, involving phosphorylation of Akt, through cytoplasmic tail of the receptor containing the consensus tetrapeptide Asn-Pro-Xaa-Tyr (NPxY).[19,20] Activation of Akt plays a critical role in controlling survival and apoptosis.[21–23] It has been reported that TG-rich lipoproteins from type IV hyperlipidemic patients induce phosphorylation of p38 MAPK, CREB and IkBα, and activate DNA binding activity of transcriptional factors, CREB, NF-κB and AP-1 in endothelial cells.[24] In addition, treatment with LDL on smooth muscle cells results in the activation of protein kinase C and MAP kinase as well as the induction of the cell cycle-related genes, c-*fos*, c-*myc* and early growth response gene-1.[25]

We found that LDLR mRNA expression in non-tumorous part of intestinal mucosa in Min mice was higher compared to those of wild-type mice, suggesting the high efficiency of intestinal lipid absorption in Min mice. Certain tumors, such as human colorectal carcinomas,[26] and human lung adenocarcinoma cell lines A549,[27] express huge amount of LDLR. It has been shown that COX-2 is up-regulated in colorectal adenocarcinomas specimens, having overexpressed LDLR mRNA compared with normal mucosa.[28] Our present data also showed that COX-2 was expressed in large quantities in the polyp stroma near the luminal surface where LDLR was up-regulated. Moreover, it has been demonstrated that PGE_2 produced by stromal cells affects on epithelial cells through EP_2 receptor.[29] Taken together, we postulate that the intestinal polyps in the Min mice with high levels of COX-2 expression respond to inappropriate uptake of fatty acids through LDLR. However, further experiments are needed to clarify a functional relationship between LDLR and COX-2.

In conclusion, this study indicated that lipid accumulation and overexpression of LDLR in polyp epithelium may contribute to polyp development. Thus, LDLR is suggested to be a potential target site for chemoprevention of colon cancer. As it is becoming increasingly clear that high fat diet, obesity and hyperlipidemia are important players in carcinogenesis, our observations may lead to a better understanding of the role of lipid accumulation in colon carcinogenesis.

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Original Contribution

Visceral Fat Volume and the Prevalence of Colorectal Adenoma

Taiki Yamaji*, Motoki Iwasaki, Shizuka Sasazuki, Norie Kurahashi, Michihiro Mutoh, Shuji Yamamoto, Masahiro Suzuki, Noriyuki Moriyama, Keiji Wakabayashi, and Shoichiro Tsugane

* Correspondence to Dr. Taiki Yamaji, Epidemiology and Prevention Division, Research Center for Cancer Prevention and Screening, National Cancer Center, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan (e-mail: tyamaji@ncc.go.jp).

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Few epidemiologic investigations of visceral adiposity and colorectal neoplasms have attempted the direct quantification of visceral fat. The authors measured visceral fat volume among middle-aged and elderly Japanese men and women who underwent colonoscopy and positron emission tomography/computed tomography for cancer screening in Tokyo, Japan, between February 2004 and February 2005, and examined the association between visceral adiposity and colorectal adenoma in 1,205 eligible subjects. Odds ratios and 95% confidence intervals for colorectal adenoma were estimated by using an unconditional logistic regression model after adjustment for potential confounders. Despite its high correlation with body mass index, visceral fat volume was associated with the prevalence of colorectal adenoma independently of body mass index in both sexes. After further adjustment for body mass index, the odds ratio of colorectal adenoma for the highest compared with the lowest quartile of visceral fat volume was 1.58 (95% confidence interval: 1.11, 2.24) for men and women combined. Conversely, body mass index was unlikely to modify the association between visceral fat volume and colorectal adenoma ($P_{interaction} = 0.39$). These findings add to accumulating evidence that visceral adiposity exerts an important influence on the pathogenesis of colorectal neoplasms. The mechanisms of this potential association between visceral adiposity and colorectal carcinogenesis warrant further investigation.

adenoma; case-control studies; intestine, large; intra-abdominal fat; Japan

Abbreviation: CI, confidence interval.

Obesity is associated with a large number of chronic diseases, including diabetes mellitus, hypertension, cardiovascular disease, and even some types of cancer (1). Of importance, a growing body of evidence suggests that the health risks of obesity relate to not only the amount of total body fat but also the distribution of body fat in the visceral region (1, 2). Visceral adiposity is a major determinant of insulin resistance, which has been suggested to be an early and fundamental disorder in the path to these obesity-related diseases (2).

In the last decade, a number of epidemiologic studies have reported an increase in the risk of colorectal adenoma (3–6) and cancer (3, 7–10) associated with a higher waist/hip ratio or waist circumference. Although these anthropometric measurements serve as surrogate markers of visceral adiposity, they mainly reflect the distribution of body fat in the abdominal region without distinction between visceral and subcutaneous fat (11). In fact, although direct quantification of visceral fat requires imaging techniques, such as computed tomography and magnetic resonance imaging (11), we are aware of only 3 epidemiologic investigations of colorectal neoplasms that have used imaging techniques (12–14). Moreover, visceral adiposity in these studies was evaluated by the visceral fat area from a single abdominal cross-section only, and no previous study has measured the visceral fat volume of the abdominal cavity, which is the most comprehensive assessment of visceral adiposity.

Colorectal adenoma is recognized as a precursor lesion to the majority of colorectal cancers, and its presence is associated with an increased risk of colorectal cancer (15). Here, we quantified the visceral fat area and volume among middle-aged and elderly Japanese men and women using

Table 1. Selected Characteristics of Cases and Controls by Sex, the Colorectal Adenoma Study in Tokyo, Japan, 2004–2005

Characteristic	Men					Women				
	Cases (N = 432)		Controls (N = 373)		$P_{difference}$[a]	Cases (N = 205)		Controls (N = 195)		$P_{difference}$[a]
	No.	%[b]	No.	%[b]		No.	%[b]	No.	%[b]	
Age category, years					0.18					0.98
40–49						13	6	13	7	
50–54	61	14	69	19		23	11	25	13	
55–59	116	27	108	29		53	26	50	26	
60–64	114	26	95	25		68	33	61	31	
≥65	141	33	101	27		48	23	46	24	
Smoking status					<0.001					0.10
Never smoker	122	28	127	34		162	79	169	87	
Past smoker	202	47	200	54		28	14	19	10	
Current smoker	108	25	46	12		15	7	7	4	
Drinking status					0.19					0.16
Never drinker	45	10	50	13		98	48	96	49	
Past drinker	22	5	26	7		10	5	3	2	
Current drinker	365	84	297	80		97	47	96	49	
Family history of colorectal cancer					0.93					0.001
Yes	57	13	50	13		46	22	21	11	
No	375	87	323	87		159	78	174	89	
NSAID use					0.009					0.59
Yes	18	4	32	9		12	6	14	7	
No	414	96	341	91		193	94	181	93	
	Median	Interquartile Range	Median	Interquartile Range		Median	Interquartile Range	Median	Interquartile Range	
Total energy intake, kcal/day	1,931	1,601–2,350	1,945	1,570–2,279	0.75	1,826	1,465–2,159	1,778	1,369–2,078	0.12
Physical activity, MET-hours/day	33.8	30.9–38.5	33.7	31.6–37.7	0.51	35.5	31.8–42.5	36.1	31.3–42.0	0.98
Height, cm	167	163–171	167	163–171	0.65	155	151–158	154	151–158	0.31
Body mass index, kg/m^2	24.0	22.4–25.7	23.5	21.7–25.1	0.008	22.5	20.6–24.2	21.8	20.3–23.6	0.09
Visceral fat area, cm^2	99.5	75.9–130	92.6	67.1–119	0.005	70.9	51.5–91.3	68.2	49.2–88.2	0.25
Visceral fat volume, cm^3	3,632	2,698–4,756	3,274	2,296–4,145	<0.001	2,123	1,505–2,801	1,880	1,390–2,575	0.02

Abbreviations: MET, metabolic equivalent task; NSAID, nonsteroidal antiinflammatory drug.
[a] Based on the chi-square test for percentage difference and the Wilcoxon rank-sum test for median difference.
[b] Percentages may not add to 100% because of rounding.

multislice computed tomography, and we examined the association between visceral adiposity and the prevalence of colorectal adenoma.

MATERIALS AND METHODS

Study population

The Research Center for Cancer Prevention and Screening was established in 2004 as a branch of the National Cancer Center of Japan with the goal of developing and evaluating preventive methods, screening modalities, and screening programs for various types of cancers. Among its efforts, the Research Center conducted the Colorectal Adenoma Study in Tokyo (16, 17), a case-control study specifically designed to investigate environmental and genetic factors related to the early stage of colorectal carcinogenesis among healthy volunteer examinees of a colorectal cancer screening. All examinees gave written, informed consent to allow their data and materials collected through the screening to be used for medical research. The study protocol was approved by the institutional review board of the National Cancer Center, Tokyo, Japan.

Table 2. Association of Body Mass Index, Visceral Fat Area, and Visceral Fat Volume With Colorectal Adenoma, the Colorectal Adenoma Study in Tokyo, Japan, 2004–2005

Measurement	Quartile									
	1					2				
	Range	No. of Cases	No. of Controls	Odds Ratio	95% Confidence Interval	Range	No. of Cases	No. of Controls	Odds Ratio	95% Confidence Interval
Body mass index										
Men, kg/m^2	<21.7	77	93			21.7–<23.5	102	93		
Crude estimate[a]				1	Referent				1.33	0.88, 2.02
Adjusted estimate[b]				1	Referent				1.36	0.88, 2.09
Women, kg/m^2	<20.3	40	47			20.3–<21.8	45	50		
Crude estimate[a]				1	Referent				1.07	0.60, 1.94
Adjusted estimate[b]				1	Referent				1.03	0.55, 1.93
Men and women combined		117	140				147	143		
Crude estimate[a]				1	Referent				1.24	0.89, 1.74
Adjusted estimate[b,c]				1	Referent				1.25	0.89, 1.77
Visceral fat area										
Men, cm^2	<67.1	76	93			67.1–<92.6	113	93		
Crude estimate[a]				1	Referent				1.49	0.99, 2.24
Adjusted estimate[b]				1	Referent				1.41	0.92, 2.16
Women, cm^2	<49.2	45	48			49.2–<68.2	53	49		
Crude estimate[a]				1	Referent				1.15	0.64, 2.04
Adjusted estimate[b]				1	Referent				1.28	0.68, 2.38
Men and women combined		121	141				166	142		
Crude estimate[a]				1	Referent				1.36	0.98, 1.91
Adjusted estimate[b,c]				1	Referent				1.30	0.92, 1.84
Visceral fat volume										
Men, cm^3	<2,296	77	93			2,296–<3,274	101	93		
Crude estimate[a]				1	Referent				1.33	0.88, 2.01
Adjusted estimate[b]				1	Referent				1.29	0.84, 1.99
Women, cm^3	<1,390	34	48			1,390–<1,880	53	49		
Crude estimate[a]				1	Referent				1.63	0.89, 3.02
Adjusted estimate[b]				1	Referent				1.86	0.96, 3.60
Men and women combined		111	141				154	142		
Crude estimate[a]				1	Referent				1.41	0.99, 1.98
Adjusted estimate[b,c]				1	Referent				1.39	0.98, 1.97

Table continues

Eligible subjects were defined in advance as men aged 50–79 years and women aged 40–79 years who underwent total colonoscopy from the anus to the cecum and who were without a history of colorectal adenoma, any malignant neoplasm, ulcerative colitis, Crohn's disease, familial adenomatous polyposis, carcinoid tumor, or colectomy. Of a consecutive series of 3,212 examinees undergoing magnifying colonoscopy with indigo carmine dye spraying between February 2004 and February 2005, 2,234 met these conditions. On the basis of the pit pattern of colorectal lesions, namely, the characteristics of mucosal crypts, 526 men and 256 women were determined to have at least 1 adenomatous polyp and were thus included as adenoma cases. Pit-pattern classification based on magnifying chromoendoscopy has been detailed elsewhere (18). Of the remaining 1,452 examinees, 482 men and 721 women were identified as potential controls who were also free from other benign lesions (e.g., hyperplastic polyps, inflammatory polyps, and diverticula). For efficiency, 256 of the potential female controls were frequency matched to the female cases in 5 age categories (40–49, 50–54, 55–59, 60–64, and ≥65 years of age) and 2 screening periods (first

Table 2. Continued

Quartile										
3					4					P_{trend}
Range	No. of Cases	No. of Controls	Odds Ratio	95% Confidence Interval	Range	No. of Cases	No. of Controls	Odds Ratio	95% Confidence Interval	
23.5–<25.1	102	93			≥25.1	151	94			
			1.37	0.90, 2.07				1.98	1.33, 2.95	0.001
			1.40	0.91, 2.15				1.99	1.31, 3.03	0.001
21.8–<23.6	53	49			≥23.6	67	49			
			1.28	0.71, 2.30				1.64	0.92, 2.92	0.07
			1.47	0.78, 2.79				1.79	0.97, 3.31	0.03
	155	142				218	143			
			1.34	0.96, 1.88				1.85	1.33, 2.57	<0.001
			1.36	0.96, 1.93				1.88	1.34, 2.63	<0.001
92.6–<119	103	93			≥119	140	94			
			1.40	0.92, 2.12				1.80	1.20, 2.69	0.01
			1.29	0.84, 1.99				1.61	1.06, 2.46	0.05
68.2–<88.2	49	49			≥88.2	58	49			
			1.07	0.58, 1.95				1.26	0.70, 2.29	0.50
			1.17	0.61, 2.24				1.36	0.72, 2.58	0.42
	152	142				198	143			
			1.25	0.89, 1.76				1.58	1.14, 2.21	0.01
			1.18	0.83, 1.67				1.46	1.03, 2.06	0.06
3,274–<4,145	89	93			≥4,145	165	94			
			1.18	0.77, 1.80				2.13	1.43, 3.16	<0.001
			1.14	0.74, 1.76				2.02	1.33, 3.05	0.001
1,880–<2,575	47	49			≥2,575	71	49			
			1.45	0.77, 2.72				2.21	1.19, 4.08	0.02
			1.66	0.84, 3.30				2.55	1.31, 4.95	0.01
	136	142				236	143			
			1.24	0.87, 1.76				2.11	1.51, 2.94	<0.001
			1.21	0.85, 1.73				2.03	1.44, 2.86	<0.001

[a] Controlled for matching variables only.
[b] Additionally adjusted for cigarette smoking, alcohol drinking, physical activity, height, total energy intake, family history of colorectal cancer, and nonsteroidal antiinflammatory drug use.
[c] Further adjusted for sex.

and second halves). Because there were fewer potential male controls than male cases, all potential male controls were included in the study. Finally, the study enrolled 782 cases and 738 controls. Cases with adenomatous polyps of ≥5 mm in diameter were referred to clinical hospitals, including the National Cancer Center, for definitive diagnosis and treatment.

Visceral fat and anthropometric measurements

Among the study subjects, 1,214 (640 cases, 574 controls) underwent positron emission tomography/computed tomography as an additional cancer screening. Subjects were scanned in the supine position with a 16-detector row computed tomography scanner (Toshiba Medical

Table 3. Association of Visceral Fat Area and Visceral Fat Volume With Colorectal Adenoma After Adjustment for Body Mass Index, the Colorectal Adenoma Study in Tokyo, Japan, 2004–2005

Residual	Quartile																P_{trend}
	1				2				3				4				
	No. of Cases	No. of Controls	Odds Ratio	95% Confidence Interval	No. of Cases	No. of Controls	Odds Ratio	95% Confidence Interval	No. of Cases	No. of Controls	Odds Ratio	95% Confidence Interval	No. of Cases	No. of Controls	Odds Ratio	95% Confidence Interval	
Residuals of visceral fat area[a]																	
Men	114	93			85	93			98	93			135	94			
Adjusted estimate[b]			1	Referent			0.68	0.45, 1.04			0.82	0.54, 1.26			0.98	0.65, 1.49	0.85
Women	43	48			66	49			43	49			53	49			
Adjusted estimate[b]			1	Referent			1.28	0.70, 2.33			0.93	0.49, 1.76			1.05	0.56, 1.97	0.84
Men and women combined	157	141			151	142			141	142			188	143			
Adjusted estimate[b,c]			1	Referent			0.90	0.64, 1.25			0.84	0.60, 1.18			0.99	0.71, 1.40	0.92
Residuals of visceral fat volume[a]																	
Men	87	93			91	93			110	93			144	94			
Adjusted estimate[b]			1	Referent			1.07	0.70, 1.63			1.29	0.84, 1.97			1.59	1.04, 2.45	0.02
Women	35	48			55	49			51	49			64	49			
Adjusted estimate[b]			1	Referent			1.56	0.83, 2.93			1.45	0.77, 2.74			1.90	1.00, 3.61	0.08
Men and women combined	122	141			146	142			161	142			208	143			
Adjusted estimate[b,c]			1	Referent			1.20	0.85, 1.69			1.28	0.90, 1.80			1.58	1.11, 2.24	0.01

[a] Residuals of visceral fat area and visceral fat volume were derived from Willett's residual method (Willett WC. *Nutritional Epidemiology*, 2nd ed. New York, NY: Oxford University Press; 1998) (22) and were uncorrelated with body mass index.

[b] Adjusted for cigarette smoking, alcohol drinking, physical activity, height, total energy intake, family history of colorectal cancer, and nonsteroidal antiinflammatory drug use, as well as matching variables.

[c] Further adjusted for sex.

Systems, Tochigi, Japan) and the following scan conditions: 120 kV, scanning time of 0.5 second/rotation, and computed tomography automatic exposure control with a standard deviation of 20. The scanner computer reconstructed the raw data to give cross-sectional images with a 5-mm slice thickness by using a function for abdominal kernel.

The visceral fat area and volume were quantified by using specifically designed software (Fujifilm Medical, Tokyo, Japan), with the case and control status unknown. A function of the software enables the automatic drawing of a contour line around the abdominal cavity, which helps to separate the subcutaneous and intraabdominal regions. Details of the software have been described elsewhere (19). To measure visceral fat volume, we first selected a series of reconstruction images from immediately below the diaphragmatic surface of the heart to the cranial edge of the pubic symphysis, minimizing the influence of pericardial and mediastinal adipose tissue. The visceral fat area was then computed in each image by electronically determining pixels with attenuation values between −190 and −30 Hounsfield units in the intraabdominal region. Finally, the visceral fat volume was calculated by integrating these visceral fat areas of respective images with a 5-mm slice thickness.

Body weight and height were measured by medical personnel at the beginning of cancer screening. Body mass index was calculated (weight (kg)/height $(m)^2$). The waist/hip ratio was not calculated because waist and hip circumferences were not measured during the study period.

Self-administered questionnaire

Prior to cancer screening, examinees completed a self-administered questionnaire that included items on personal medical history, present medication, family history of cancer, cigarette smoking, alcohol drinking, physical activity, and other lifestyle factors. The questionnaire has been described in detail elsewhere (16, 17).

The questionnaire also included a food frequency questionnaire of 145 food and beverage items with standard portions/units and 9 frequency categories. Total energy intake was estimated from the responses by reference to the *Standard Tables of Food Composition in Japan*, Fifth Revised Edition (20). The food frequency questionnaire of the present study was essentially the same as that used in a large prospective study among a Japanese population (21).

Statistical analysis

An unconditional logistic regression model was used to estimate odds ratios and their 95% confidence intervals of colorectal adenoma according to sex-specific quartiles of body mass index, visceral fat area, and visceral fat volume, with the lowest quartile for each measurement used as the reference. The first analysis controlled for matching variables (i.e., age categories and screening periods), while the second additionally adjusted for the following covariates: cigarette smoking (never, ≤20, 21–40, >40 pack-years), alcohol drinking (never, past, <150, 150–299, ≥300 g per week), family history of colorectal cancer (yes or no), and nonsteroidal antiinflammatory drug use (yes or no). These covariates were suggested to be potential confounders in previous reports from the Colorectal Adenoma Study in Tokyo (16, 17). The second analysis also adjusted for components or surrogate indicators of energy balance, namely, total energy intake in kilocalories per day, physical activity in metabolic equivalent hours per day, and height in centimeters. These continuous variables were, respectively, divided into quartiles, the cutoff points of which were based on the sex-specific distribution among controls. Linear trends in the odds ratios of colorectal adenoma were also assessed by assigning ordinal values to quartiles of each measurement. Finally, we combined men and women according to sex-specific quartiles of body mass index, visceral fat area, and visceral fat volume, respectively, and examined whether the association of each measurement was modified by sex. An interaction term was created by multiplying ordinal values for quartiles of each measurement by those for dichotomous categories of sex, and its significance was statistically evaluated by the likelihood ratio test with 1 df.

In a preliminary analysis, the visceral fat area and visceral fat volume were found to be highly correlated with body mass index in both sexes, with Pearson's correlation coefficients of 0.66 and 0.74, respectively, for male controls and 0.66 and 0.72, respectively, for female controls (all P values < 0.001). These strong correlations notwithstanding, we were interested to know whether the visceral fat area and visceral fat volume were associated with colorectal adenoma independently of body mass index. To investigate this, we applied Willett's residual method for energy adjustment of food intake (22) and calculated residuals of the visceral fat area and visceral fat volume using linear regression models with body mass index as the independent variable. These residuals were uncorrelated with body mass index, which enabled us to evaluate the association of visceral fat area and visceral fat volume with colorectal adenoma after adjustment for body mass index.

Of 1,214 subjects who underwent both colonoscopy and positron emission tomography/computed tomography, 9 had missing information, namely, 3 with regard to body mass index, 2 for tobacco smoking, and 4 for physical activity. These were then excluded, and the current analysis was conducted in 805 men (432 cases, 373 controls) and 400 women (205 cases, 195 controls). Two-sided P values of less than 0.05 were regarded as statistically significant. All statistical analyses were carried out by using Statistical Analysis System (SAS), Version 9.1, software (SAS Institute, Inc., Cary, North Carolina).

RESULTS

The visceral fat volume was evaluated from immediately below the diaphragmatic surface of the heart to the cranial edge of the pubic symphysis, while the visceral fat area was examined at the level of the umbilicus. Table 1 shows that the degree of visceral adiposity differed considerably between men and women, with the median visceral fat volume approximately 74% greater in male than female controls. Table 1 also summarizes selected characteristics of cases and controls

Table 4. Association of Visceral Fat Area and Visceral Fat Volume With Colorectal Adenoma Stratified by Body Mass Index, the Colorectal Adenoma Study in Tokyo, Japan, 2004–2005

Measurement	Quartile									
	1					2				
	Range	No. of Cases	No. of Controls	Odds Ratio	95% Confidence Interval	Range	No. of Cases	No. of Controls	Odds Ratio	95% Confidence Interval
Visceral fat area[a]										
Men, cm^2	<67.1					67.1–<92.6				
Women, cm^2	<49.2					49.2–<68.2				
Lower body mass index[c]		107	125				88	82		
Adjusted estimate[d]				1	Referent				1.16	0.77, 1.76
Higher body mass index[c]		14	16				78	60		
Adjusted estimate[d]				1.15	0.52, 2.55				1.55	0.99, 2.41
Visceral fat volume[a]										
Men, cm^3	<2,296					2,296–<3,274				
Women, cm^3	<1,390					1,390–<1,880				
Lower body mass index[c]		102	126				92	87		
Adjusted estimate[d]				1	Referent				1.28	0.85, 1.92
Higher body mass index[c]		9	15				62	55		
Adjusted estimate[d]				0.72	0.29, 1.78				1.45	0.91, 2.31

Table continues

by sex. Current smokers were more prevalent among male cases than controls, whereas nonsteroidal antiinflammatory drug use was more common among male controls than cases. On the other hand, a family history of colorectal cancer was more frequent among female cases than controls.

Table 2 shows the odds ratios of colorectal adenoma according to sex-specific quartiles of body mass index, visceral fat area, and visceral fat volume. We observed a statistically significant trend of increasing adjusted odds ratios for colorectal adenoma across quartiles of body mass index in both men and women (P_{trend} = 0.001 and 0.03, respectively). A statistically significant odds ratio was also seen among men in the highest quartile. Adjusted odds ratios of colorectal adenoma for the highest compared with the lowest quartile of body mass index were 1.99 (95% confidence interval (CI): 1.31, 3.03) for men and 1.79 (95% CI: 0.97, 3.31) for women. Of the 2 measurements of visceral adiposity, an increase in visceral fat volume was related to a higher prevalence of colorectal adenoma, with a significant trend in both sexes (P_{trend} = 0.001 and 0.01 for men and women, respectively), whereas the visceral fat area was not associated with colorectal adenoma in either sex (P_{trend} = 0.05 and 0.42 for men and women, respectively). Adjusted odds ratios of colorectal adenoma for the highest compared with the lowest quartile of visceral fat area and visceral fat volume were 1.61 (95% CI: 1.06, 2.46) and 2.02 (95% CI: 1.33, 3.05), respectively, for men and 1.36 (95% CI: 0.72, 2.58) and 2.55 (95% CI: 1.31, 4.95), respectively, for women. When men and women were combined according to sex-specific quartiles of each measurement, a significant trend of increasing adjusted odds ratios across quartiles became more pronounced for body mass index and visceral fat volume (P_{trend} < 0.001 for both), while the nonsignificant trend for visceral fat area remained (P_{trend} = 0.06). Despite the sex differences in body mass and visceral adiposity, significant effect modification by sex was not observed for any of 3 measurements ($P_{interaction}$ = 0.75, 0.30, and 0.87 for body mass index, visceral fat area, and visceral fat volume, respectively; data not shown).

To investigate the influence of visceral fat area and visceral fat volume after adjustment for body mass index, we calculated residuals of visceral fat area and visceral fat volume against body mass index, which were uncorrelated with body mass index. As shown in Table 3, no material association was seen between visceral fat area and colorectal adenoma after adjustment for body mass index. In contrast, visceral fat volume was related to a higher prevalence of colorectal adenoma even after adjustment for body mass index. Adjusted odds ratios of colorectal adenoma for the highest compared with the lowest quartile of residuals of visceral fat area and visceral fat volume were 0.99 (95% CI: 0.71, 1.40) and 1.58 (95% CI: 1.11, 2.24), respectively, for men and women combined. Further, a positive trend across quartiles was statistically significant (P_{trend} = 0.01) for visceral fat volume. Again, significant effect modification by sex was not observed for either measurement of visceral adiposity ($P_{interaction}$ = 0.53 and 0.83 for visceral fat area and visceral fat volume, respectively; data not shown). These residual analyses revealed that visceral fat volume

Table 4. Continued

Quartile										
	3				4					P_{trend}
Range	No. of Cases	No. of Controls	Odds Ratio	95% Confidence Interval	Range	No. of Cases	No. of Controls	Odds Ratio	95% Confidence Interval	
										0.71[b]
92.6–<119					≥119					
68.2–<88.2					≥88.2					
	47	56				22	20			
			0.88	0.54, 1.43				1.08	0.54, 2.17	0.89
	105	86				176	123			
			1.41	0.94, 2.10				1.53	1.06, 2.20	0.70
										0.39[b]
3,274–<4,145					≥4,145					
1,880–<2,575					≥2,575					
	43	55				27	15			
			0.90	0.54, 1.48				2.05	1.01, 4.17	0.24
	93	87				209	128			
			1.35	0.89, 2.03				1.95	1.36, 2.79	0.01

[a] Men and women were combined according to sex-specific quartiles of each measurement of visceral adiposity.
[b] Values are $P_{interaction}$ instead of P_{trend}.
[c] Body mass index was dichotomized on sex-specific median values for controls (23.5 and 21.8 kg/m^2 for men and women, respectively).
[d] Adjusted for cigarette smoking, alcohol drinking, physical activity, height, total energy intake, family history of colorectal cancer, and nonsteroidal antiinflammatory drug use, as well as sex and matching variables.

was associated with the prevalence of colorectal adenoma independently of body mass index. Further adjustment for body mass index on a continuous scale did not essentially change these results (data not shown).

We then examined whether body mass index modified the association of visceral fat area and visceral fat volume with colorectal adenoma (Table 4). In this analysis, men and women were combined according to sex-specific quartiles of each measurement of visceral adiposity and stratified by body mass index based on sex-specific median values for controls. Compared with men and women with a lower body mass index as well as in the lowest quartile of visceral adiposity, those with a higher body mass index as well as in the highest quartile had a significantly higher prevalence of colorectal adenoma, with adjusted odds ratios of 1.53 (95% CI: 1.06, 2.20) and 1.95 (95% CI: 1.36, 2.79) for the visceral fat area and visceral fat volume, respectively. In addition, the adjusted odds ratio was also statistically significant among those with a lower body mass index but in the highest quartile of visceral fat volume (odds ratio = 2.05, 95% CI: 1.01, 4.17). On the other hand, when comparison was made among those with a higher body mass index, adjusted odds ratios of colorectal adenoma for the highest compared with the lowest quartile of visceral fat area and visceral fat volume were 1.33 (95% CI: 0.60, 2.92) and 2.69 (95% CI: 1.11, 6.55), respectively (data not shown). Importantly, statistical evaluation of interaction between visceral adiposity and body mass index revealed that body mass index was unlikely to modify the association of visceral fat area or visceral fat volume with colorectal adenoma, either ($P_{interaction} = 0.71$ and 0.39 for visceral fat area and visceral fat volume, respectively). When the above analysis was conducted for men and women separately, results were essentially the same.

DISCUSSION

In this study, we directly quantified the degree of visceral adiposity among middle-aged and elderly Japanese men and women and observed that, although visceral fat volume was highly correlated with body mass index, an increase in the visceral fat volume was associated with a higher prevalence of colorectal adenoma independently of body mass index in both sexes. Conversely, body mass index was unlikely to modify the association between visceral fat volume and colorectal adenoma.

To our knowledge, this is the first study to examine the association of visceral fat volume with any type of colorectal neoplasm. In contrast, 3 epidemiologic studies have investigated the association between visceral fat area and

colorectal adenoma (12–14); of these, 2 demonstrated a statistically significant positive association, albeit with relatively small study sizes ($n \leq 200$) (12, 14), whereas the third found no association in a larger, but still small, population ($n = 458$) (13). Although this apparent inconsistency may simply reflect sparse data from a limited number of studies or chance due to the small number of subjects, another likely contributor is that all 3 studies combined men and women, with different male/female ratios. As shown in the present study, however, the degree of visceral adiposity differs considerably between men and women. Moreover, the influence of visceral adiposity on risk may differ by sex. Further studies with a sufficient number of men and women to enable sex-specific analyses are needed.

Several mechanisms that implicate visceral adiposity in colorectal carcinogenesis have been hypothesized. One well-known hypothesis is that visceral adiposity may be associated with factors that promote the growth of colorectal adenomas, thereby increasing the risk of colorectal cancer. Visceral adiposity is, in fact, a strong determinant of insulin resistance and subsequent hyperinsulinemia (2), while insulin is an important growth factor for colonic mucosal cells and colonic carcinoma cells in vitro (23) and may have the potential to mediate the association between visceral adiposity and colorectal neoplasms.

Computed tomography allows the accurate quantification of visceral fat and is presently the optimum technique in this regard (11). However, there are several disadvantages associated with the use of this technique as an assessment tool of visceral adiposity in practical settings, including health hazards of exposure to ionizing radiation. On the other hand, although the waist/hip ratio and waist circumference provide inexact measurements of visceral adiposity, they are not only safe but also cheap and relatively easy to perform (11). These anthropometric measurements therefore remain useful for the general classification of large numbers of people by visceral adiposity.

Among the strengths of the present study, the provision of total colonoscopy to all study subjects would have decreased the likelihood of misclassification between cases and controls. Moreover, the number of subjects was by far larger than in previous colorectal neoplasm studies that evaluated visceral adiposity by using computed tomography (12–14). Finally, case-control studies of colorectal adenoma have a methodological advantage over those of colorectal cancer, in that bias related to changes in body composition caused by the cancer itself is avoided.

A major limitation of this study is that colorectal adenomas were identified by magnifying colonoscopy with indigo carmine dye spraying, which may have resulted in some misclassification among adenoma cases. Given that pit-pattern classification based on magnifying chromoendoscopy differentiates colorectal lesions with approximately 90% accuracy (24), however, and that our institution has reported accuracy in differential diagnosis of ≥95% (25), the influence of this misclassification on the present results is likely to have been minimal. A second limitation is the relatively small body size of the study population. For male and female controls, the median body mass index was 23.5 and 21.8 kg/m^2, respectively, and the prevalence of overweight and obesity was 28% and 13%, respectively. Our findings may not therefore be directly applicable to severely obese populations, often found in North American and European countries, where more than half of adults are overweight or obese (1). Further studies in populations with larger body sizes are thus required. Third, because of their lower prevalence of colorectal adenoma, sample sizes for women were relatively small, and in some cases significant results could not be obtained because of limited statistical power. Nonetheless, statistical evaluation of interaction between visceral adiposity and sex revealed that, despite an obvious sex difference in the degree of visceral adiposity, its positive association with colorectal adenoma was likely to be similar between sexes. Finally, the present study was based on not incident but prevalent cases, meaning that the odds ratios of colorectal adenoma presented in this study did not necessarily indicate the risk of "developing" colorectal adenoma. Rather, they represent the risk of "having" colorectal adenoma at a point in time, and they should therefore be interpreted with caution.

In summary, with an optimal technique for the direct quantification of visceral fat, our present study corroborates previous findings obtained by using inexact surrogate markers of visceral adiposity, namely, waist/hip ratio and waist circumference. Our findings add to accumulating evidence that visceral adiposity exerts an important influence on the pathogenesis of colorectal neoplasms. The mechanisms of this potential association between visceral adiposity and colorectal carcinogenesis warrant further investigation.

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Author affiliations: Epidemiology and Prevention Division, Research Center for Cancer Prevention and Screening, National Cancer Center, Tokyo, Japan (Taiki Yamaji, Motoki Iwasaki, Shizuka Sasazuki, Norie Kurahashi, Shoichiro Tsugane); Screening Technology and Development Division, Research Center for Cancer Prevention and Screening, National Cancer Center, Tokyo, Japan (Shuji Yamamoto, Masahiro Suzuki, Noriyuki Moriyama); and Cancer Prevention Basic Research Project, National Cancer Center Research Institute, Tokyo, Japan (Michihiro Mutoh, Keiji Wakabayashi).

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