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Treatment with Anti-Interleukin-6 Receptor Antibody Ameliorates Intestinal Polyposis in *Apc*$^{Min/+}$ Mice under High-Fat Diet Conditions

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The prevalence of colorectal malignancies is increasing in the world. The parallel increase of metabolic syndrome gives a speculation between these two conditions, although the precise mechanism is still unclear. Interleukin-6 (IL-6) is a cytokine known to correlate with obesity and serve as a proinflammatory adipokine. In the present study, we investigated the effect of IL-6 signaling blockade on intestinal polyp formation in obesity using a mouse model of adenomatous polyposis coli (Apc). Male C57BL/6J-*Apc*$^{Min/+}$ mice were fed a high-fat diet from 5 weeks of age, and the overweight mice thus obtained were given a weekly intraperitoneal injection of anti-mouse IL-6 receptor antibody (MR16-1) from 6 to 15 weeks of age, while control mice received IgG or phosphate-buffered saline (PBS). The total number of intestinal polyps was significantly decreased in the MR16-1-injected group (53.1 ± 6.8) relative to the control groups (PBS-injected, 81.3 ± 6.1; rat IgG-injected, 74.7 ± 4.8, p = 0.01), and in particular the number of polyps larger than 2 mm in diameter was markedly decreased. In addition, the mean diameter of polyps in the MR16-1-injected group was significantly smaller than that in the control groups. On the other hand, no significant differences in body weight, epididymal fat pad mass, or the plasma levels of glucose, insulin and triglyceride were observed among the three groups. Thus, treatment with anti-IL-6 receptor antibody suppressed polyp growth in obese *Apc*$^{Min/+}$ mice fed the high-fat diet. We suggest that IL-6 signaling may be responsible for the obesity-associated colorectal tumorigenesis.

Keywords: anti-interleukin-6 receptor antibody; colorectal cancer; inflammation; insulin resistance; metabolic syndrome
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Introduction

Colorectal cancer is a common and highly frequent cause of cancer death worldwide (Weitz et al. 2005). During the past few decades, particularly in Asian countries, there has been a marked increase in the incidence of colorectal cancer, most likely because of the adoption of a Western-style diet associated with increased intake of animal fat, and a reduction of physical activity levels (Sung et al. 2005). It is now generally accepted that most colorectal cancers develop from colorectal adenomas and show morphological and genetic progression through an adenoma-carcinoma sequence (Fearon and Vogelstein 1990). Based on this theory, it is reasonable to consider that colorectal adenomas and carcinomas should have similar epidemiological features and share a common etiology.

Several studies have indicated that metabolic syndrome increases the risk for both colorectal adenoma and cancer by approximately 50% (Kahi et al. 2008; Matthews et al. 2010; Sato et al. 2011). We have demonstrated that an increased area of visceral fat and a decreased concentration of plasma adiponectin are associated with the development of colorectal adenoma (Otake et al. 2005) and early cancer (Otake et al. 2010). Although the mechanisms underlying

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this association remain unclear, insulin resistance and subsequently hyperinsulinemia, in close association with visceral fat accumulation, are thought to be important etiologic factors (Giovannucci and Michaud 2007).

It has been recognized that adipose tissue is not only a reservoir for surplus energy, but also an active endocrine organ that contributes to metabolic homeostasis by secreting several adipokines such as adiponectin, leptin, tumor necrosis factor-α, interleukin-6 (IL-6), macrophage and monocyte chemoattractant protein-1, and plasminogen activator inhibitor-1 (Kershaw and Flier 2004). These adipokines are able to induce a chronic state of low-grade inflammation that is involved in the pathogenesis of obesity-related insulin resistance (Bastard et al. 2006). Chronic inflammation is also thought to be associated with colorectal carcinogenesis (Terzic et al. 2010). Recently, in a cross-sectional case-control study, we showed that circulating serum levels of IL-6 were increased in male individuals with colorectal adenoma, and associated with the presence of colorectal adenoma independently of insulin resistance or insulin (Sasaki et al. 2012). These findings suggest that IL-6 may provide an important link between obesity and colorectal adenoma.

IL-6 is known to be the major proinflammatory adipokine (Eder et al. 2009), and activates signal transducer and activator of transcription 3 (STAT3), thereby enhancing cell growth and stimulating the production of growth factor (Chiba et al. 2012). In the C57BL/6J-*Apc*$^{Min/+}$ (Min) mouse, which harbors a point mutation in the *adenomatous polyposis coli* (*Apc*) gene and has been used extensively as a colon cancer model, IL-6 was shown to stimulate the proliferation of premalignant enterocytes (Terzic et al. 2010). Thus, IL-6 appears to have a direct role in promoting the development of colorectal adenoma.

In the present study, we therefore investigated whether inhibition of IL-6 signaling by treatment with MR16-1 (rat anti-mouse IL-6 receptor monoclonal antibody) influences tumorigenesis in Min mice fed a high-fat diet. The specific blockade of IL-6 signaling with MR16-1 has been well confirmed in previous studies (Tamura et al. 1993; Takagi et al. 1998; Katsume et al. 2002; Tomiyama-Hanayama et al. 2009).

Materials and Methods

Animals

Min mouse progenitors were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). Male Min mice were mated with wild-type female C57BL/6J mice to establish a breeding colony. Genotyping to distinguish Min and wild-type mice was performed by polymerase chain reaction (PCR) using genomic DNA isolated from the tail, following the recommendations of the Jackson Laboratory.

Four to six male mice were housed per metal cage with sterilized softwood chips as bedding, in a specific pathogen-free animal room air-conditioned at 24 ± 2°C and 55% humidity, under a 12-hour light:dark cycle. A basal diet (20% protein, 55% carbohydrate, and 4.5% fat, Picolab Rodent Diet 20, Picolab, Richmond, Indiana) and tap water were available *ad libitum*. From 5 weeks of age, these mice were fed a high-fat diet (20% protein, 35% carbohydrates, and 45% fat, Rodent Diet, Research Diets Inc., NJ, USA) until the end of the study. The animals were observed daily for clinical signs and morbidity, and body weights and food consumption were measured weekly. This study was conducted after receiving approval from the Animal Center Ethical Review Committee in the Faculty of Medicine, Yamagata University.

Experimental protocols

First, to investigate the effect of a high-fat diet on intestinal polyp formation, male Min mice at 5 weeks of age were fed a high-fat diet for 11 weeks, and samples of the intestinal tract and plasma were collected at 16 weeks of age (Fig. 1A). Control Min mice were fed a basal diet.

Second, at 6 weeks of age, the male Min mice were randomly divided into three groups (9 mice/group): phosphate-buffered saline (PBS)-injected group, rat IgG-injected group (class-matched control,

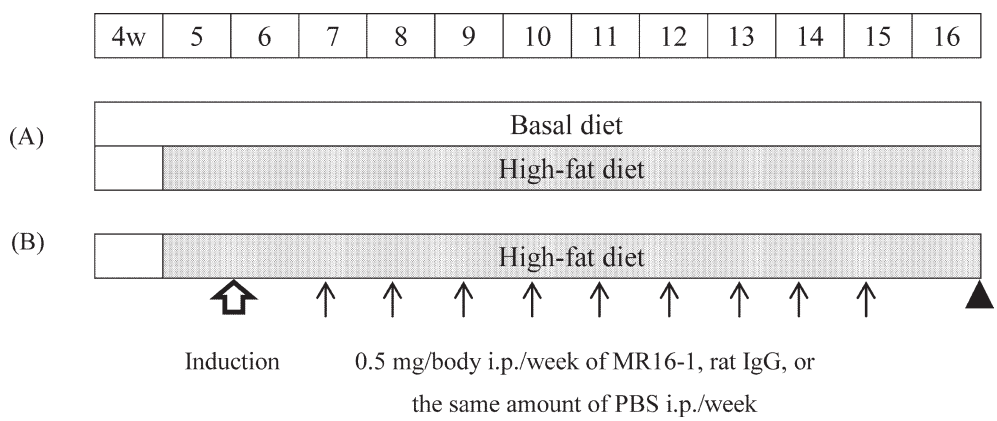

Fig. 1. Experimental protocols.
(A) Male *Apc*$^{Min/+}$ mice at 5 weeks of age were fed the hig-fat diet for 11 weeks (n = 10), whereas male Min mice were fed a basal diet (n = 15). (B) Male Min mice were first injected intraperitoneally with 2 mg/body MR16-1, or control rat IgG antibody at 6 weeks of age to induce tolerance against rat IgG; mice in the PBS group were injected with the same volume of PBS. MR16-1-injected group (n = 9), rat IgG-injected group (n = 9), and PBS-injected group (n = 9). All mice were euthanatized with sevoflurane at 16 weeks of age after an overnight fast.

MP Biomedicals, OH, USA), or MR16-1-injected group (rat anti-mouse IL-6 receptor monoclonal antibody, Chugai Pharmaceutical Co., Ltd., Tokyo, Japan). The specificity and blocking ability of this monoclonal antibody have been well confirmed in previous reports (Tamura et al. 1993; Takagi et al. 1998; Katsume et al. 2002; Okazaki et al. 2002).

Mice were first injected intraperitoneally with 2 mg/body MR16-1, or control rat IgG antibody at 6 weeks of age to induce tolerance against rat IgG (Katsume et al. 2002); mice in the PBS group were injected with the same volume of PBS. Subsequent injections were performed with 0.5 mg/body antibodies, or the same volume of PBS weekly from 7 to 15 weeks of age (Fig. 1B).

All mice were euthanatized with sevoflurane at 16 weeks of age after an overnight fast.

Tissue sample collection and polyp counts

The epididymal fat, and intestinal tract were removed. The small intestine was divided into the proximal segment (4 cm in length), and proximal (middle) and distal halves of the remainder. The intestines were opened longitudinally and fixed flat between filter paper in 10% buffered formalin. The numbers of polyps and their largest diameters, and their distributions in the intestine, were assessed with a stereoscopic microscope.

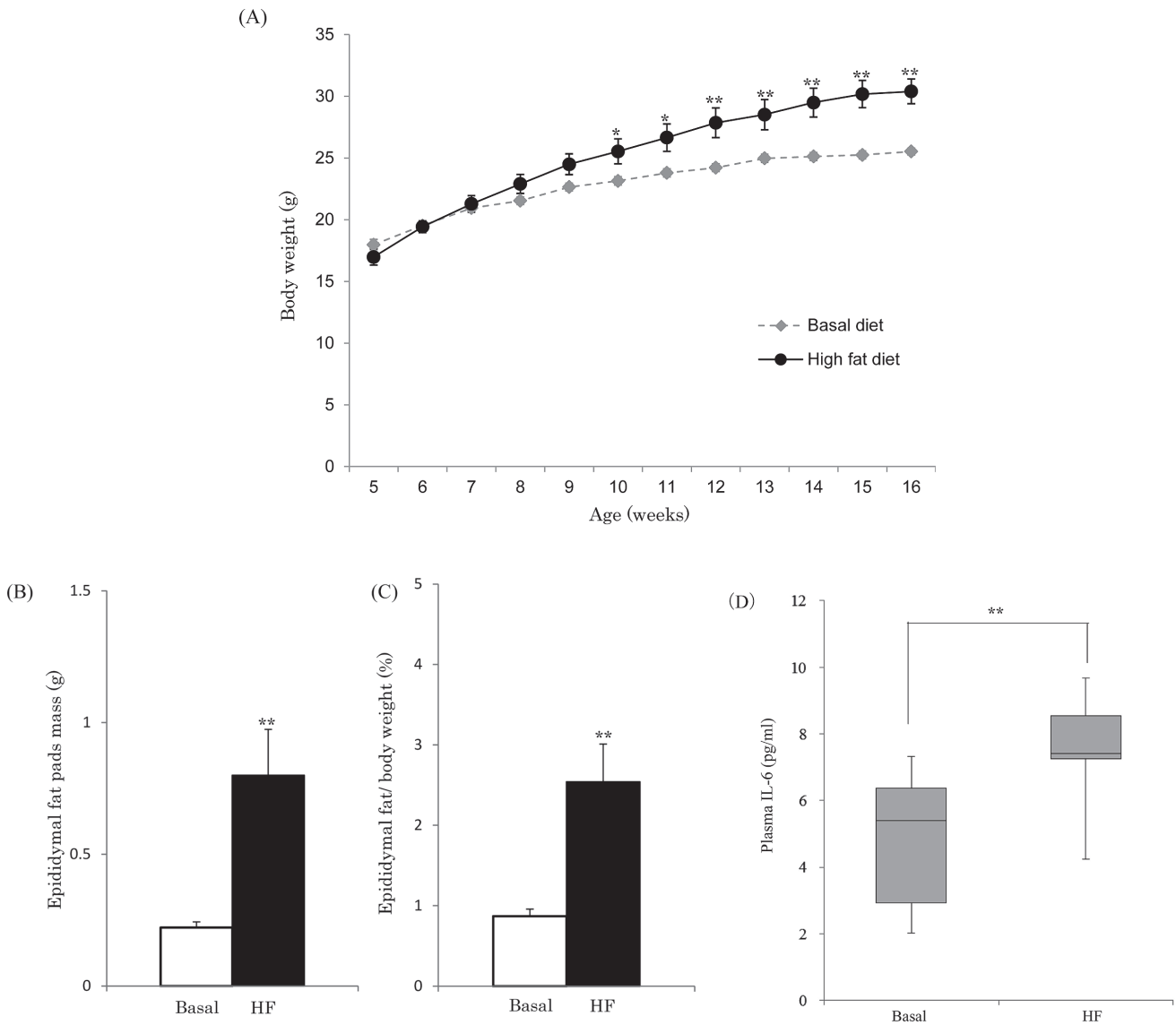

Fig. 2. Features of Min mice fed the high-fat diet
(A) Body weight of *Apc*$^{Min/+}$ mice was measured every week from the age of 5 weeks to 16 weeks. Data are shown as means ± SE. *$p < 0.05$, **$p < 0.01$ compared to mice on the basal diet by 2-way repeated-measures ANOVA. (B) Epididymal fat pads mass and (C) epididymal fat/body weight at 16 weeks of age. White bars: basal diet group (Basal, $n = 15$), black bars: high-fat diet group (HF, $n = 10$). Each bar represents the mean with the SE. **$p < 0.01$ compared to mice on the basal diet by Student's t test. (D) Plasma level of IL-6 at 16 weeks of age. White bars: basal diet group (Basal, $n = 15$), black bars: high-fat diet group (HF, $n = 10$). Each bar represents the mean with the SE. **$p < 0.01$ compared to mice on the basal diet by Wilcoxon signed-rank test.

Blood sample collection and plasma measurements

Blood samples were collected from the abdominal vein at autopsy after an overnight fast. These samples were then placed immediately into tubes containing ethylenediaminetetra-acetic acid (EDTA). Plasma samples were stored at −80°C. Plasma concentrations of glucose, triglyceride, insulin, and IL-6 were measured using glucose CII test Wako, triglyceride E-test Wako (Wako, Osaka, Japan), Mouse Insulin ELISA kit (Shibayagi, Shibukawa, Japan), and Mouse IL-6 immunoassay ELISA kit (R&D Systems, Inc., USA), respectively.

Statistical analysis

Data are presented as mean ± standard error (SE) unless otherwise noted. Student's *t* test or Wilcoxon signed-rank test was used to compare the median values between two groups. One-way ANOVA or Kruskal-Wallis test was used to determine significance among three groups, and subsequent post hoc analyses were performed using Dunnet's method. Differences at a probability (p) value of < 0.05 were considered to be significant. We carried out all statistical calculations using SAS Enterprise Guide v. 4.3 (SAS Institute, Inc., NC, USA).

Results

Promotion of intestinal polyp formation in Min mice under a high-fat diet

Compared to the basal diet group, mean body weight was significantly higher in the Min mice fed the high-fat diet from 10 to 16 weeks, and the final weights were 25.5 ± 0.2 g and 30.3 ± 1.0 g, respectively ($p < 0.01$, Fig. 2A). At

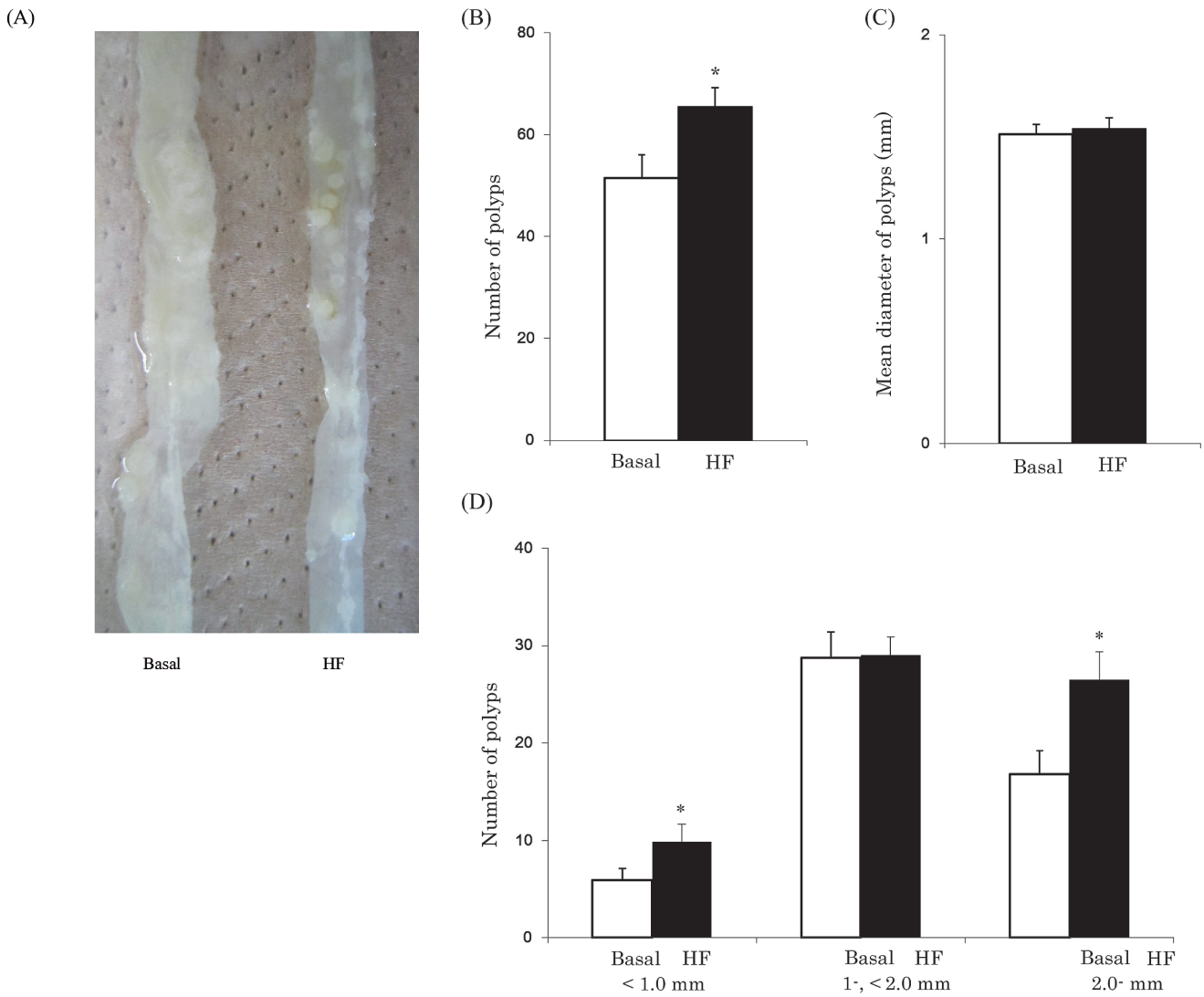

Fig. 3. Promotion of intestinal polyp formation in Min mice fed the high-fat diet.
(A) Representative photograph of the small intestinal polyps at 16 weeks of age. Photograph was taken at the similar part of distal portion of small intestine, which was freshly opened longitudinally. Number (B) and mean size (C) of small-intestinal polyps in *Apc*$^{Min/+}$ mice at 16 weeks of age. Data are shown as means ± SE. (D) Distribution of the mean number of small-intestinal polyps of each size. White bars: basal diet group (Basal, $n = 15$), black bars: high-fat diet group (HF, $n = 10$). Each bar represents the mean with the SE. *$p < 0.05$ compared to mice on the basal diet by Student's *t* test.

16 week of age, epididymal fat pad mass (0.2 ± 0.02 vs. 0.8 ± 0.2 g, $p < 0.01$, Fig. 2B and 0.8 ± 0.1 g/body weight% vs. 2.5 ± 0.4 g/body weight%, $p < 0.01$, Fig. 2C). The median levels of plasma IL-6 (5.4 pg/ml, IQR: interquartile range 2.67-6.41 vs. 7.4 pg/ml, IQR 7.21-8.76, $p = 0.01$, Fig. 2D) were significantly higher in the Min mice fed the high-fat diet.

The total number of polyps was significantly increased in the Min mice fed the high-fat diet (65.4 ± 3.8) in comparison with the control group (51.4 ± 4.5, $p = 0.02$, Fig. 3A, B). No significant difference in mean polyp diameter was observed between the groups (Fig. 3C). The number of small polyps less than 1 mm (5.9 ± 1.1 vs. 9.9 ± 1.7, $p = 0.04$) or more than 2 mm (16.8 ± 2.4 vs. 26.5 ± 2.8, $p <$

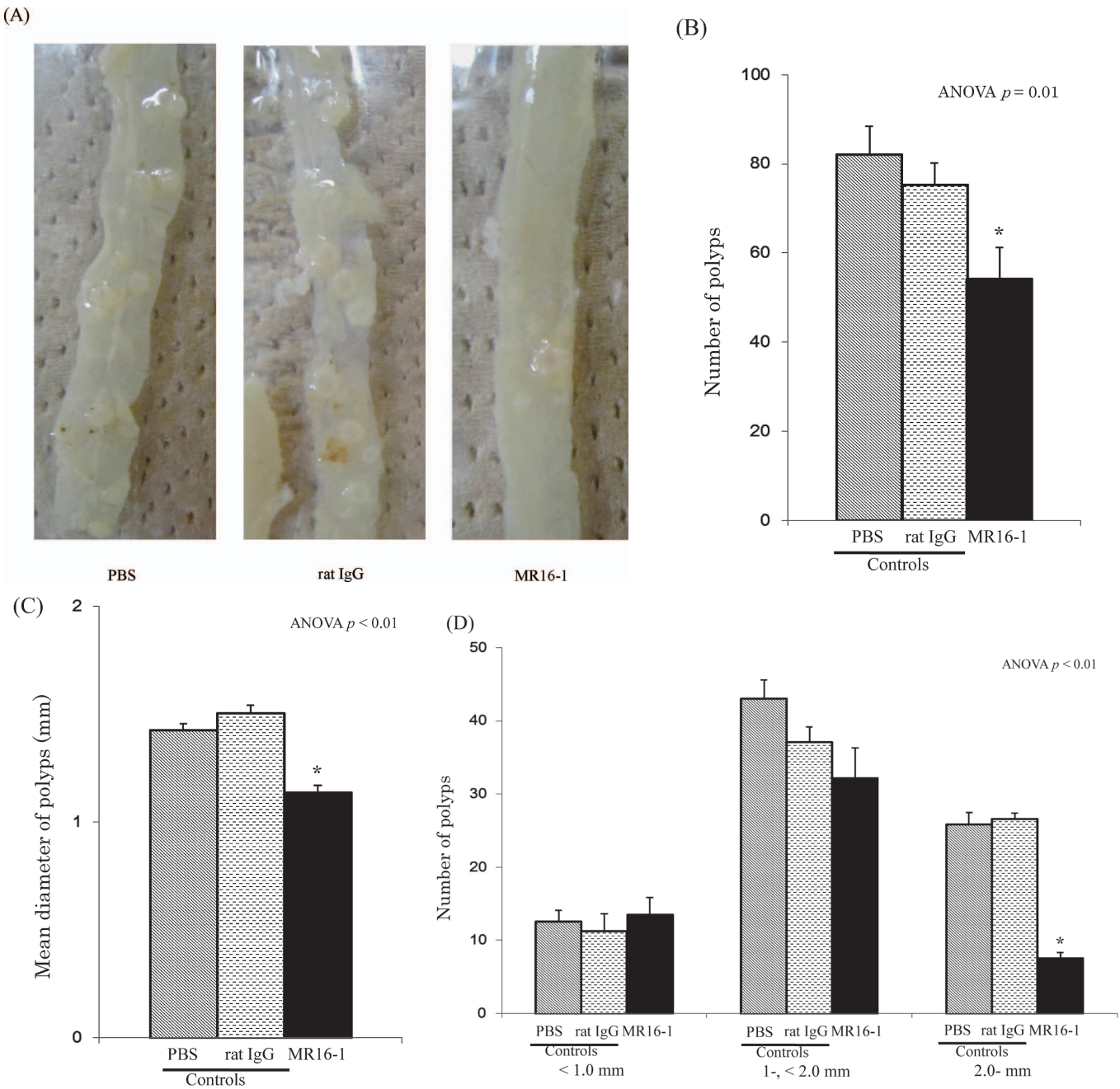

Fig. 4. MR16-1 administration ameliorates intestinal polyp formation in Min mice fed the high-fat diet.
(A) Representative photographs of the small intestinal polyps in 16 weeks age male Min mice treated with PBS, rat IgG and MR16-1. Photographs were taken at the similar part of distal portion of small intestine, which were opened longitudinally. Number (B) and mean size (C) of small intestinal polyps in $Apc^{Min/+}$ mice at 16 weeks of age. Data are shown as means ± SE. (D) Distribution of the mean number of small-intestinal polyps of each size. PBS-injected group ($n = 9$), rat IgG-injected group ($n = 9$), and MR16-1-injected group ($n = 9$). Each bar represents the mean with the SE. *$p <$ 0.01 compared to control groups by Dunnet's test.

Table 1. Plasma parameters in *Apc*$^{Min/+}$ mice.

Group	Glucose (mg/dl)				Insulin (ng/ml)			Triglyceride (mg/dl)		
	N	median	IQR	*p*	median	IQR	*p*	median	IQR	*p*
PBS-injected	9	192	166-249	0.13	0.60	0.42-1.64	0.23	56.7	51.5-101.6	0.10
rat IgG-injected	9	200	165-224		0.52	0.20-1.05		128	81.3-198.1	
MR16-1-injected	9	163	159-167		1.41	0.66-1.54		56.7	18.3-123.0	

IQR, interquartile range.

0.01) in diameter was also increased in the Min mice fed the high-fat diet (Fig. 3D).

Intestinal polyp formation following MR16-1 administration in Min mice fed the high-fat diet

The body weights in the three groups of Min mice were similar during the study period, and the mean final weights were 31.0 ± 0.8 g (MR16-1-injected group), 30.2 ± 0.9 g (rat IgG-injected group), and 31.3 ± 0.9 g (PBS-injected group). Average weekly food intake and epididymal fat pad mass did not differ significantly among the three groups (data not shown). The total number of polyps was significantly lower in the MR16-1-injected group (53.1 ± 6.8) than in the control groups (PBS-injected, 81.3 ± 6.1; rat IgG-injected, 74.7 ± 4.8, $p = 0.01$, Fig. 4A, B). The mean diameter of polyps was significantly smaller in the MR16-1-injected group (1.10 ± 0.03 mm) than in the controls (PBS-injected, 1.41 ± 0.02 mm; rat IgG-injected, 1.48 ± 0.03 mm, $p < 0.01$, Fig. 4C). The number of large polyps more than 2 mm diameter was markedly decreased in the MR16-1-injected group (7.5 ± 1.1) in comparison with the controls (PBS-injected, 25.7 ± 2.6; rat IgG-injected, 26.5 ± 1.5, $p < 0.01$, Fig. 4D).

Changes in plasma levels of glucose, insulin and triglyceride after MR16-1 administration in Min mice fed the high-fat diet

In an attempt to clarify the mechanisms affecting development of intestinal polyps, we examined the plasma levels of glucose, insulin and triglyceride. However, no significant differences in these parameters were observed among the three groups (Table 1).

Discussion

In the present study, we demonstrated that the number of intestinal polyps and plasma levels of IL-6 were significantly increased in obese Min mice fed a high-fat diet, and that exogenous administration of anti-mouse IL-6 receptor antibody suppressed the development of intestinal polyps in those mice, without affecting nutritional conditions, or the plasma levels of insulin, glucose and triglyceride. To our knowledge, no previous study has investigated the effect of MR16-1 on colorectal carcinogenesis.

It is well known that inflammatory bowel disease is an important risk factor for the development of colon cancer. Several experimental and clinical studies have shown that the major cytokine IL-6 is linked to the pathogenesis of inflammation-associated colorectal cancer (Grivennikov et al. 2010; Waldner et al. 2012). In mice with complete IL-6 deficiency exposed to AOM (azoxymethane) + DSS (dextran sodium sulfate), which are widely used as a model of colitis-associated cancer, reduced tumor development has been reported (Grivennikov et al. 2009). Thus, IL-6 seems to act as a critical mediator of tumor development, especially in a detectable inflammatory state.

On the other hand, subclinical, often undetectable inflammation, such as that induced by obesity, may increase the risk of cancer (Grivennikov et al. 2010). Obesity is a known risk factor for colorectal cancer and adenoma (Otake et al. 2005; Giovannucci and Michaud 2007). IL-6 is increased in obese, relative to lean, individuals (Fantuzzi 2005). It has been shown that adipose tissue releases IL-6 (Weisberg et al. 2003), which can induce chronic low-grade inflammation. These factors appear to be consistent with our present observations of an increased circulating level of IL-6 and tumor promotion in obese Min mice.

It is known that an elevated level of IL-6 is linked to the development of insulin resistance (Galic et al. 2010), which is likely involved in colorectal carcinogenesis (Chan et al. 2011). However, we have shown that serum levels of IL-6 are slightly increased in patients with adenoma, and positively associated with the risk of adenoma, independently of insulin or insulin resistance (Sasaki et al. 2012). In the present study, we showed that treatment with anti-IL-6 receptor antibody reduced both the number and mean diameter of intestinal polyps in Min mice fed a high-fat diet, without any difference in nutritional conditions or the plasma levels of insulin, glucose and triglyceride. These findings support our hypothesis that IL-6 may have a direct role in promoting the development of colorectal adenoma under conditions of obesity-related low-grade inflammation.

The number of polyps formed in control mice is not consistent between the two cohorts (Fig. 3B vs. Fig. 4B); namely, the number of polyps observed in PBS-treated group or IgG-treated group was larger than the number of polyps detected in high-fat diet group of the first cohort. Since no significant difference in the numbers of polyps between PBS and IgG groups was observed, we speculate that the observed difference might be due to the administering stimulation (intraperitoneal administration for once a week).

In mice fed with control diets, plasma IL-6 levels were significantly lower than those of high-fat diets fed mice (their median values: 5.4 vs. 7.4 pg/ml; see Fig. 2D). This difference may represent the effect of chronic low-grade inflammation associated with obesity. Because MR16-1 inhibits the binding of IL-6 to its receptor with high specificity, MR16-1 administration resulted in the significant decrease in the formation of polyps, despite the small difference of IL-6 level.

Several targets have been known to link to colon epithelial preneoplastic cell proliferation. The effect of IL-6 signaling in colon epithelial proliferation has been previously demonstrated in a cell line (Fenton et al. 2006). And the blockade of IL-6 signaling with the MR16-1 antibody inhibited this proliferation. Moreover IL-6 dependent growth of intestinal tumors was signaling to through downstream activation of STAT3. Past study indicates the effect of STAT3 on tumor cells was mediated the expression of various cell cycle progression regulators (Bollrath et al. 2009). Our study provides supporting evidence for the association between IL-6 signaling and intestinal neoplasia.

In conclusion, our study has demonstrated that the number of intestinal polyps and the plasma level of IL-6 were increased to a greater extent in Min mice fed a high-fat diet than in Min mice fed a basal diet, and that anti-IL-6 receptor antibody suppressed tumorigenesis in Min mice that were receiving a high-fat diet, without any significantly effects on body weight, fat mass, or the plasma levels of insulin, glucose and triglyceride. Our data suggest that IL-6 may play an active role in the etiology of colorectal tumor, and that inhibition of IL-6 signaling may suppress the effect of a high-fat diet on colorectal carcinogenesis. Further investigations are needed to clarify the precise mechanisms involved, and their clinical implications.

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Conflict of Interest

The authors declare no conflict of interest.

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