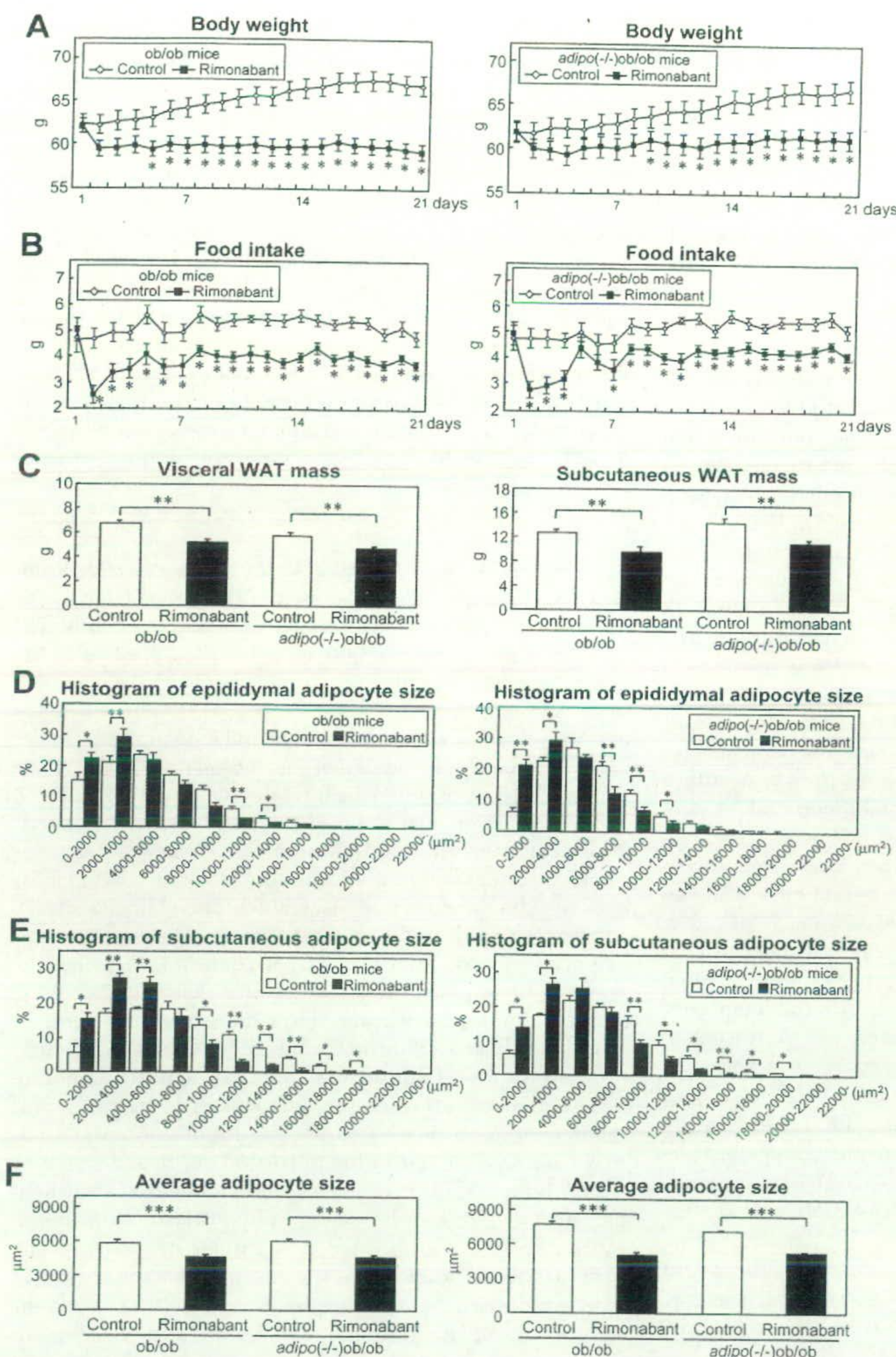

FIGURE 1. **The absence of adiponectin had no effect on rimonabant-induced suppression of body weight and daily food intake.** *A* and *B*, body weights (*A*) and food intake (*B*) of ob/ob (*left panels*) and *adipo*(−/−)ob/ob mice (*right panels*) not treated (*open squares*) and treated (*filled squares*) with rimonabant (n = 12–14/group). Values are means ± S.E. of data obtained from the analysis of ob/ob and *adipo*(−/−)ob/ob mice. *, $p < 0.05$. **, $p < 0.01$. *C*, weight of the total visceral white adipose tissue (*left panel*) and subcutaneous WAT (*right panel*) of ob/ob and *adipo*(−/−)ob/ob mice not treated (*open bars*) and treated (*filled bars*) with rimonabant (n = 9–14/group). Values are means ± S.E. of data obtained from the analysis of ob/ob and *adipo*(−/−)ob/ob mice. **, $p < 0.01$. *D* and *E*, histogram of adipocyte size from epididymal WAT (*D*) and subcutaneous WAT (*E*) of ob/ob (*left panels*) and *adipo*(−/−)ob/ob mice (*right panels*) not treated (*open bars*) and treated (*filled bars*) with rimonabant (n = 5–8/group). Values are means ± S.E. of data obtained from the analysis of ob/ob and *adipo*(−/−)ob/ob mice. *, $p < 0.05$; **, $p < 0.01$. *F*, average size of adipocyte from epididymal WAT (*left panel*) and subcutaneous WAT (*right panel*) of ob/ob and *adipo*(−/−)ob/ob mice not treated (*open bars*) and treated (*filled bars*) with rimonabant (n = 5–8/group). Values are means ± S.E. of data obtained from the analysis of ob/ob and *adipo*(−/−)ob/ob mice. ***, $p < 0.005$.

First, we measured the rectal temperature in the ob/ob and *adipo*(−/−)-ob/ob mice. The temperature was essentially the same (Fig. 2*A*), and rimonabant treatment significantly increased the rectal temperature of the ob/ob and *adipo*(−/−)ob/ob mice to a similar degree (Fig. 2*A*). Second, we investigated the oxygen consumption after 21-day treatment with rimonabant and found that in the dark phase of the daily light cycle, rimonabant increased the energy expenditure to a similar degree in both the ob/ob and *adipo*(−/−)-ob/ob mice (Fig. 2*B*). This effect of rimonabant on the energy expenditure in the ob/ob mice did not require the presence of adiponectin. We next investigated the effects of rimonabant treatment on the serum lipid levels. In addition to reducing the body weight, rimonabant has been demonstrated to reduce the serum triglyceride (TG) (15–18, 32) and free fatty acid (FFA) levels (32). However, the involvement of adiponectin in this action of rimonabant remains unclear. Both the serum TG and FFA levels were indistinguishable between the ob/ob and *adipo*(−/−)ob/ob mice (Fig. 2, *C* and *D*), and rimonabant treatment significantly decreased the levels of both to similar degrees in the ob/ob and *adipo*(−/−)ob/ob mice (Fig. 2, *C* and *D*). This effect of rimonabant on the serum lipids in the ob/ob mice did not require the presence of adiponectin. MCP-1 and resistin have been shown to be important mediators of insulin resistance linked to obesity (36–39). We analyzed the expression of MCP-1 and resistin in the epididymal WAT. The expressions of both MCP-1 and resistin were indistinguishable between the untreated and rimonabant-treated mice of either genotype (Fig. 2, *E* and *F*).

Rimonabant Increased the Plasma Adiponectin Levels in the ob/ob Mice, in Particular of High Molecular Weight Adiponectin—Rimonabant treatment for 21 days significantly increased the plasma adiponectin levels in the ob/ob mice, whereas plasma adiponectin was not detect-

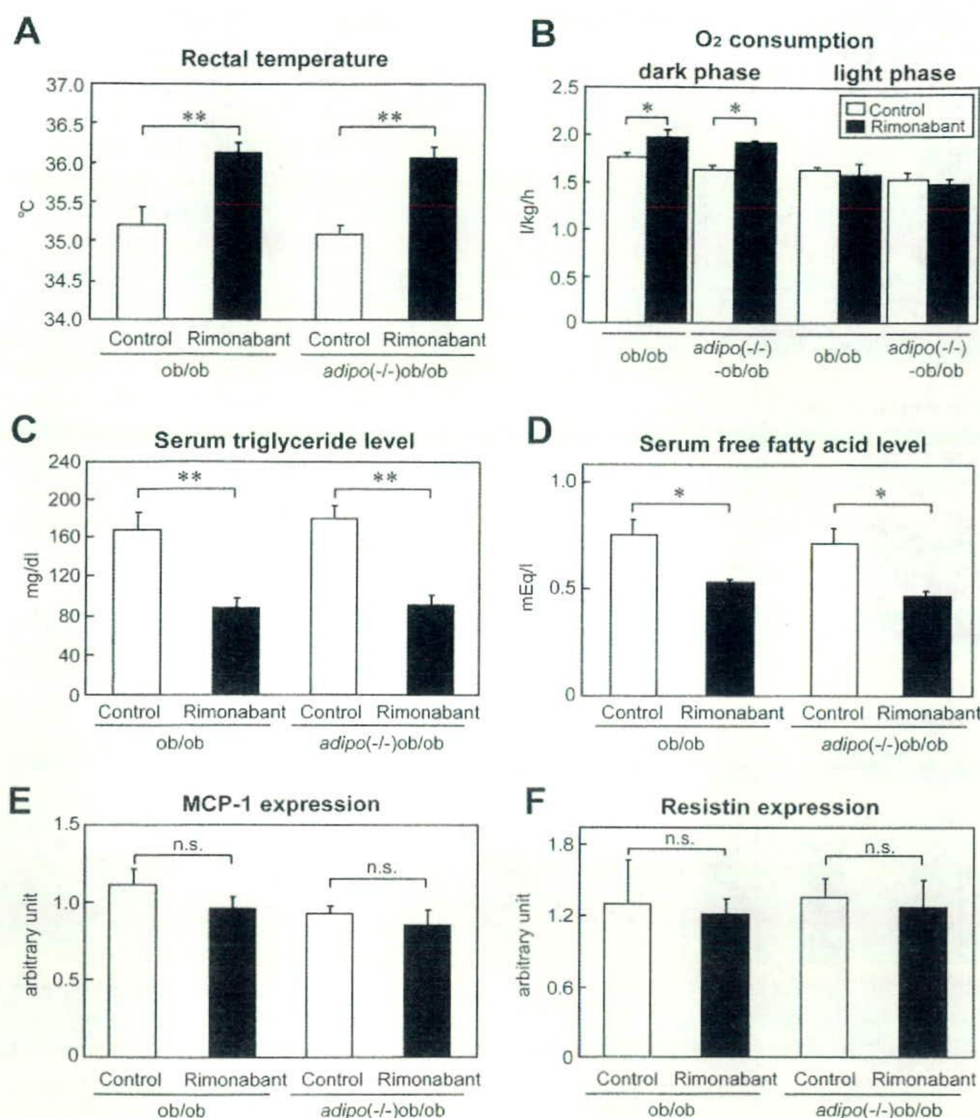

FIGURE 2. **Rimonabant increased the energy expenditure and decreased the serum triglyceride and free fatty acid levels to a similar degree in the ob/ob and *adipo*(−/−)ob/ob mice.** *A* and *B*, rectal temperature (*A*) and O_2 consumption (*B*) in ob/ob and *adipo*(−/−)ob/ob mice not treated (*open bars*) and treated (*filled bars*) with rimonabant (n = 6–10/group). Values are means ± S.E. of data obtained from the analysis of ob/ob mice and *adipo*(−/−)ob/ob mice. *, $p < 0.05$; **, $p < 0.01$. *C* and *D*, serum TG (*C*) and free fatty acid (*FFA*) (*D*) levels in ob/ob and *adipo*(−/−)ob/ob mice not treated (*open bars*) and treated (*filled bars*) with rimonabant. *C*, n = 11–14/group; *D*, n = 4–5/group. Values are means ± S.E. of data obtained from the analysis of ob/ob mice and *adipo*(−/−)ob/ob mice. *, $p < 0.05$. **, $p < 0.01$. *E* and *F*, MCP-1 (*E*) and resistin (*F*) expression levels in the epididymal WAT of ob/ob and *adipo*(−/−)ob/ob mice not treated (*open bars*) and treated (*filled bars*) with rimonabant (n = 7–8/group). Values are means ± S.E. of data obtained from the analysis of ob/ob mice and *adipo*(−/−)ob/ob mice. Values are means ± S.E. *n.s.*, not significant.

able in either the untreated or rimonabant-treated *adipo*(−/−)-ob/ob mice (Fig. 3*A*). High molecular weight (HMW) adiponectin is known to be the most active, and its serum levels have been reported to be decreased in obese individuals and murine models, which is associated with a decrease of the hepatic and muscle AMPK activity and fatty acid combustion and, thereby, exacerbation of insulin resistance (19, 20). Therefore, we analyzed the plasma levels of this isoform of adiponectin by Western blotting. Rimonabant treatment significantly increased the serum levels of HMW adiponectin in the ob/ob mice (Fig. 3*B*). On the other hand, the plasma levels of middle molecular weight and low molecular weight adiponectin were slightly, but not significantly, increased in the rimonabant-treated ob/ob mice (Fig. 3*B*). In regard to the *adipo*(−/−)-ob/ob mice, plasma adiponectin was not detectable in either the untreated or rimonabant-treated mice (Fig. 3*B*). Rimonabant has been reported to increase adiponectin expression and secretion in 3T3F442A adipocyte (6, 40). We next investigated the direct effect of rimonabant on adiponectin secretion using the murine adipocyte cell line 3T3L1 and confirmed that treatment with 100 nM and 1 μM rimonabant actually increased the expression and secretion into the medium of adiponectin (Fig. 3, *C* and *D*).

Rimonabant Improved Hepatic Insulin Resistance in both the ob/ob and adipo(−/−)ob/ob Mice, although the Effect Was Significantly Less Pronounced in the adipo(−/−)-ob/ob Mice—We carried out hyperinsulinemic-euglycemic clamp studies in the ob/ob and *adipo*(−/−)-ob/ob mice to investigate the effect of rimonabant on the insulin resistance in the liver and skeletal muscle. Without rimonabant treatment, the glucose infusion rates were comparable in the ob/ob and *adipo*(−/−)-ob/ob mice (Fig. 4*A*). After 21 days of rimonabant treatment, the glucose infusion rates were significantly increased in both the ob/ob and *adipo*(−/−)ob/ob mice (Fig. 4*A*); however, the increase was significantly less pronounced in the *adipo*(−/−)ob/ob mice. Rimonabant treatment also produced a significant decrease of the endogenous glucose production in both the ob/ob and *adipo*(−/−)ob/ob mice, but the effect was significantly less pronounced in the *adipo*(−/−)ob/ob mice (Fig. 4*B*). The rates of R_d were indistinguishable between the untreated ob/ob and *adipo*(−/−)ob/ob mice, and rimonabant treatment had no effect on this parameter in either genotype (Fig. 4*C*). We next studied the effects on insulin signaling and the downstream reactions in the liver (Fig. 4, *D* and *E*). Insulin-stimulated Akt phosphorylation was significantly increased in rimonabant-treated ob/ob mice as compared with that in the untreated ob/ob mice (Fig. 4*D*), whereas insulin-stimulated Akt phosphorylation only tended to be increased in the rimonabant-treated *adipo*(−/−)ob/ob mice as compared with that in the corresponding untreated mice. The PEPCK expression levels in the liver were comparable in the untreated ob/ob and

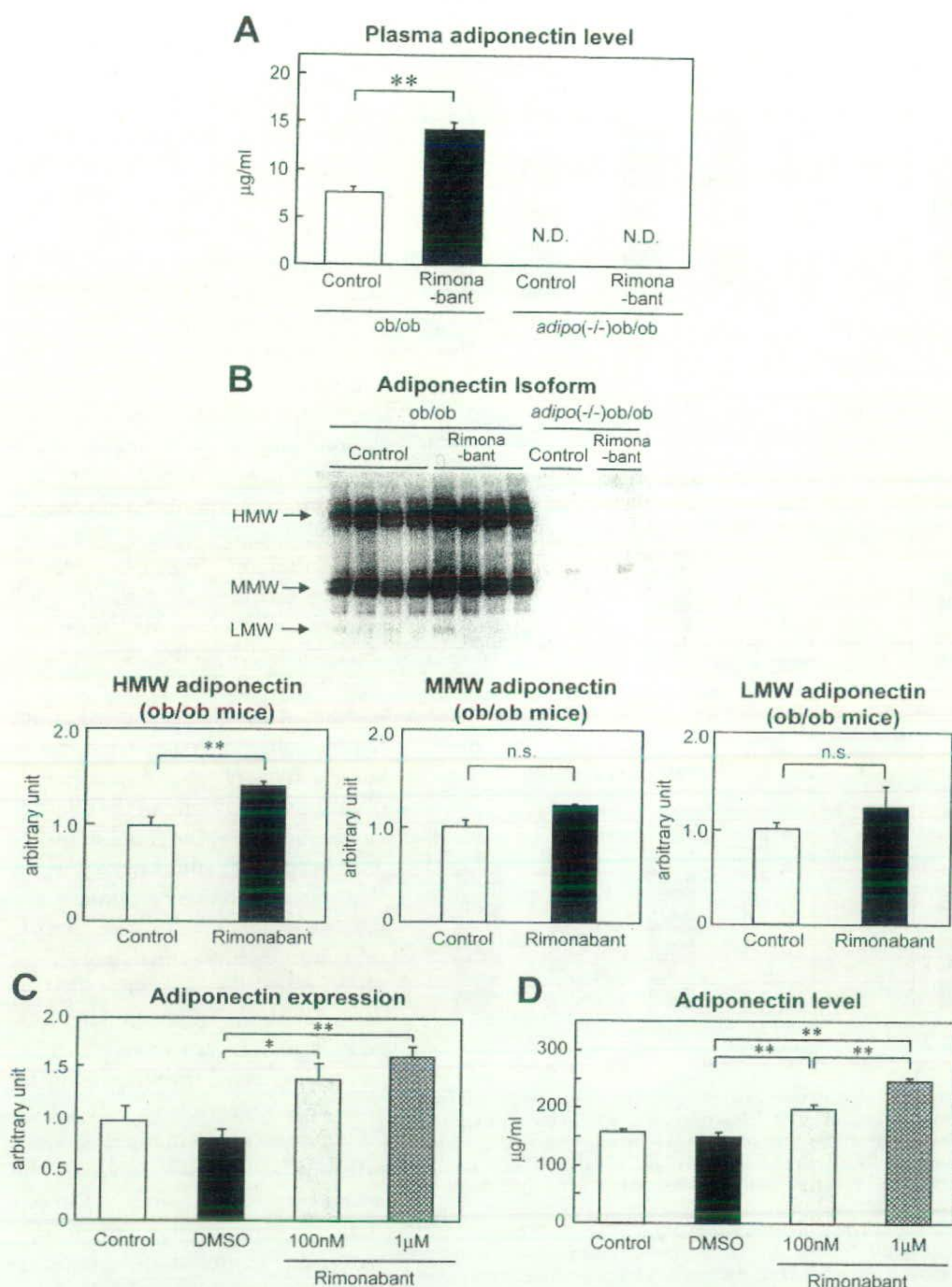

FIGURE 3. **Rimonabant increased the plasma adiponectin levels, in particular of high molecular weight adiponectin, in the ob/ob mice.** *A*, plasma adiponectin levels in ob/ob and *adipo*(−/−)ob/ob mice not treated (*open bar*) and treated (*filled bar*) with rimonabant (n = 7–14/group). Values are means ± S.E. of data obtained from the analysis of ob/ob mice and *adipo*(−/−)ob/ob mice. **, $p < 0.01$. *N.D.*, not detectable. *B*, the different isoforms of plasma adiponectin of ob/ob and *adipo*(−/−)ob/ob mice not treated (*open bars*) and treated (*filled bars*) with rimonabant were analyzed by Western blotting and quantitated by densitometry. The relative ratio of each molecular weight category of adiponectin was normalized to that in the control ob/ob mice not treated with rimonabant (n = 4–8/group). Results are representative of three independent experiments. Values are means ± S.E. of data obtained from the analysis of ob/ob mice and *adipo*(−/−)ob/ob mice. *, $p < 0.05$. *n.s.*, not significant. *C* and *D*, effects of rimonabant on adiponectin mRNA expression (*C*) and adiponectin secretion in the conditioned medium (*D*) of mouse 3T3L1 adipocytes (n = 4–9/group). Shown are controls (*open bars*), DMSO as the vehicle (*filled bars*), 100 nM rimonabant (*gray bars*), and 1 μM rimonabant (*latticed bars*). Values are means ± S.E. of data obtained from the analysis of 3T3L1 adipocytes. *, $p < 0.05$; **, $p < 0.01$.

adipo(−/−)ob/ob mice (Fig. 4*F*). Rimonabant treatment significantly decreased the expression of PEPCK in both the ob/ob and *adipo*(−/−)mice, but the effect was significantly less pronounced in the *adipo*(−/−)ob/ob mice (Fig. 4*F*). These findings indicate that rimonabant ameliorates hepatic but not muscle insulin resistance in mice with an ob/ob background, in both an adiponectin-dependent and adiponectin-independent manner.

Rimonabant Increased the Hepatic AMPK Activities and CPT-1 (Carnitine Palmitoyltransferase-1) Expression Levels in both ob/ob Mice and adipo(−/−)ob/ob Mice, but Its Effect was Significantly Less Pronounced in the adipo(−/−)ob/ob Mice—We carried out analysis of the liver metabolic activity after the clamp studies to investigate the effect of rimonabant on amelioration of insulin resistance. The AMPK activities were comparable in the untreated ob/ob and *adipo*(−/−)ob/ob mice (Fig. 5*A*). Rimonabant treatment for 21 days increased the AMPK activities in both the ob/ob and *adipo*(−/−)ob/ob mice, but its effect was significantly less pronounced in the *adipo*(−/−)ob/ob mice (Fig. 5*A*). The expression levels of CPT-1, the rate-limiting enzyme in fatty acid β-oxidation, were also comparable in the untreated ob/ob and *adipo*(−/−)ob/ob mice (Fig. 5*B*). Rimonabant treatment increased the CPT-1 expression in both ob/ob and *adipo*(−/−)ob/ob mice, but its effect was significantly less pronounced in the *adipo*(−/−)ob/ob mice (Fig. 5*B*). The expression levels of protein phosphatase 2C were indistinguishable between the untreated ob/ob and *adipo*(−/−)ob/ob mice, and rimonabant treatment had no effect on the protein phosphatase 2C expression in either genotype (Fig. 5*C*). As reported previously (26, 41), fatty acid oxidation is positively regulated by AMPK in the liver; therefore, we next carried out analysis of the hepatic TG content by Oil Red O staining. The percentage of areas of Oil Red O staining in the liver were comparable in the untreated ob/ob and *adipo*(−/−)ob/ob mice (Fig. 5*D*). Rimonabant treatment significantly decreased the hepatic TG content in both the ob/ob and *adipo*(−/−)mice, but its effect was significantly less pronounced in the *adipo*(−/−)ob/ob mice

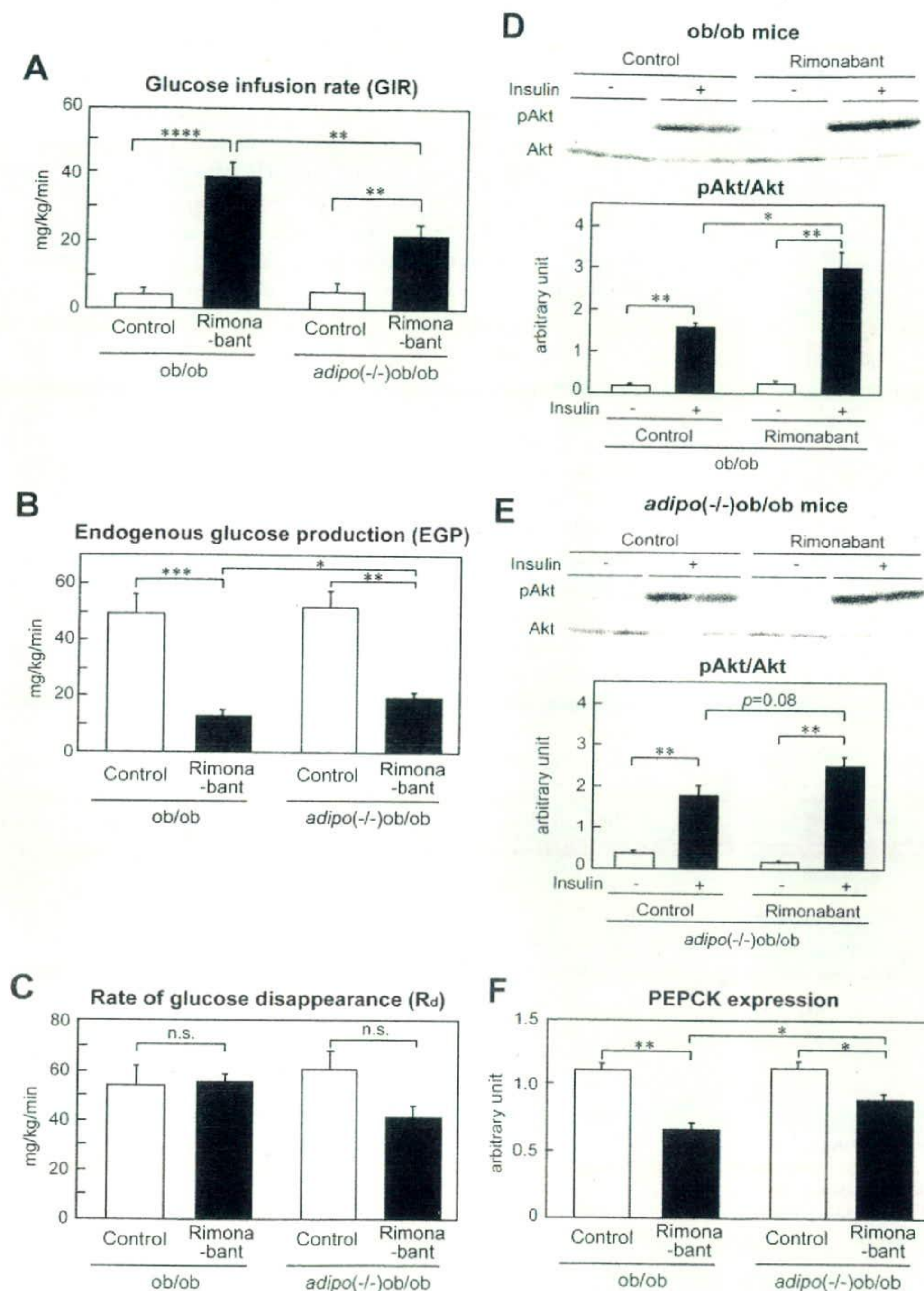

FIGURE 4. **Rimonabant improved hepatic insulin resistance in both ob/ob and *adipo*(−/−)ob/ob mice, although the effect was significantly less pronounced in the *adipo*(−/−)ob/ob mice.** *A–C*, glucose infusion rates (*GIR*) (*A*), endogenous glucose production (*EGP*) (*B*), and rates of glucose disappearance (*Rd*) (*C*) in ob/ob and *adipo*(−/−)ob/ob mice not treated (*open bars*) and treated (*filled bars*) with rimonabant in the clamp study ($n = 5–7$/group). Values are means ± S.E. of data obtained from the analysis of ob/ob mice and *adipo*(−/−)ob/ob mice. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$. *D* and *E*, phosphorylations of Akt in the livers of ob/ob (*D*) and *adipo*(−/−)ob/ob mice (*E*) not treated (*open bars*) and treated (*filled bars*) with rimonabant after the injection of insulin ($n = 4–5$/group). Results are representative of three independent experiments. Values are means ± S.E. of data obtained from the analysis of ob/ob mice and *adipo*(−/−)ob/ob mice. *, $p < 0.05$. *F*, PEPCK expression levels in the livers of ob/ob and *adipo*(−/−)ob/ob mice not treated (*open bars*) and treated (*filled bars*) with rimonabant after the clamp studies ($n = 6–7$/group). The relative expressions after normalization to the expression level of cyclophilin were compared. Values are means ± S.E. of data obtained from the analysis of ob/ob mice and *adipo*(−/−)ob/ob mice. *, $p < 0.05$; **, $p < 0.01$. pAkt, phospho-Akt. *n.s.*, not significant.

(Fig. 5*D*). We also investigated the AMPK activities in the muscle after the clamp studies. The AMPK activities in the muscle were indistinguishable between the untreated ob/ob and *adipo*(−/−)ob/ob mice, and rimonabant treatment had no effect on the muscle AMPK activity in either genotype (Fig. 5*E*). These findings indicate that rimonabant activates hepatic but not muscle AMPK in mice with an ob/ob background in both an adiponectin-dependent and adiponectin-independent manner.

DISCUSSION

The selective CB-1 blocker rimonabant has been reported to produce weight loss and ameliorate insulin resistance and metabolic abnormalities in obese animals (12, 13), as also in patients with obesity (15–18). Rimonabant has also been reported to increase the plasma adiponectin levels in animal models of obesity and diabetes, as also in diabetic or nondiabetic subjects (15, 31, 32). Adiponectin has been proposed to be a major insulin-sensitizing adipokine (19, 20) and is a plausible candidate as the adipokine mediating the rimonabant-induced amelioration of insulin resistance. Therefore, in this study, we used two obesity models, the ob/ob and *adipo*(−/−)ob/ob mice, to investigate whether the rimonabant-induced increase of plasma adiponectin might be causally involved in the insulin-sensitizing effects of the drug.

Rimonabant treatment decreased the body weight, food intake, and weight of the WAT to similar degrees in the ob/ob and *adipo*(−/−)ob/ob mice. Furthermore, it also increased the energy expenditure and decreased the serum TG and FFA to similar degrees in the ob/ob and *adipo*(−/−)ob/ob mice. Thus, the involvement of adiponectin was not requited for rimonabant to exert its effects.

Significant improvement of the insulin resistance was observed in the ob/ob mice following rimonabant treatment, in association with significant up-regulation of the plasma adiponectin levels, in particular of HMW. Amelioration of insulin resistance in the ob/ob mice was considered to be attributable to improvement of the hepatic but not muscle insulin resistance. Interestingly, these

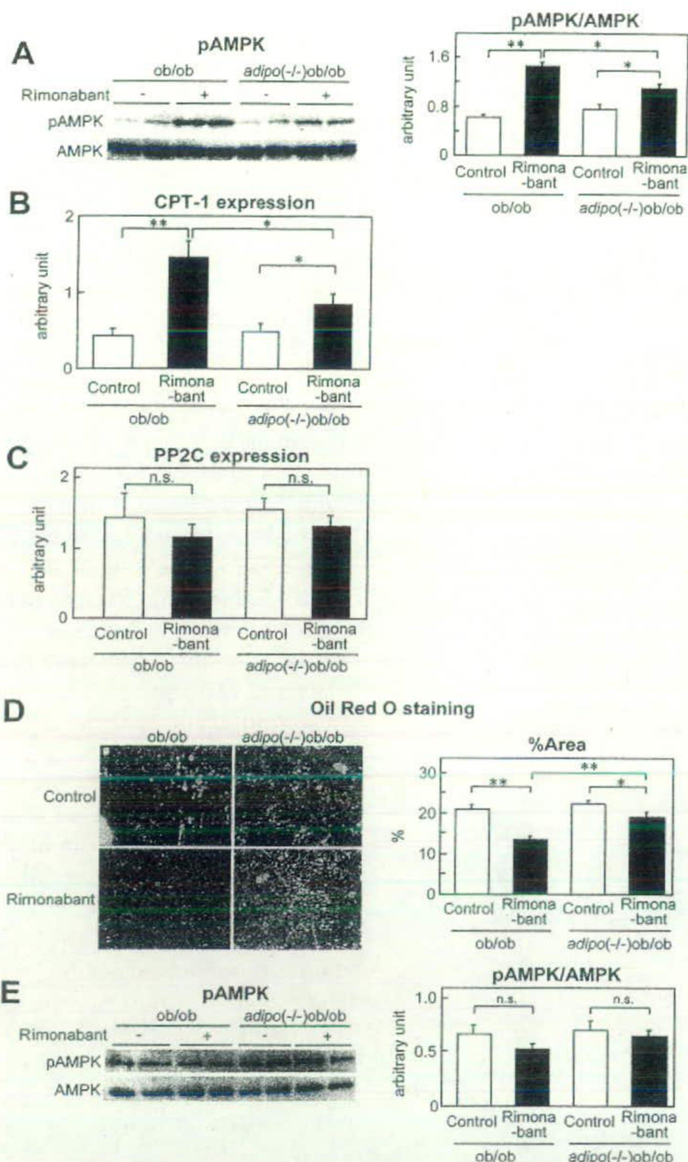

FIGURE 5. **Rimonabant increased the hepatic AMPK activities and CPT-1 expression levels in both ob/ob mice and *adipo*(−/−)ob/ob mice, but the effects were significantly less pronounced in the *adipo*(−/−)ob/ob mice.** *A*, phosphorylations of AMPK in the livers of ob/ob and *adipo*(−/−)ob/ob mice not treated (*open bars*) and treated (*filled bars*) with rimonabant after the clamp studies ($n = 4$–5/group). Results are representative of three independent experiments. Values are means ± S.E. of data obtained from the analysis of ob/ob mice and *adipo*(−/−)ob/ob mice. *, $p < 0.05$; **, $p < 0.01$. *B* and *C*, carnitine palmitoyltransferase-1 (*CPT-1*) (B) and protein phosphatase 2C (*PP2C*) (*C*) expression levels in the liver of ob/ob and *adipo*(−/−)ob/ob mice not treated (*open bars*) and treated (*filled bars*) with rimonabant after the clamp studies ($n = 4$–9/group). Relative expressions after normalization to the expression level of cyclophilin were compared. Values are means ± S.E. of data obtained from the analysis of ob/ob mice and *adipo*(−/−)ob/ob mice. *, $p < 0.05$; **, $p < 0.01$. *D*, Oil Red O staining in the livers of ob/ob and *adipo*(−/−)ob/ob mice not treated (*open bars*) and treated (*filled bars*) with rimonabant ($n = 6$–10/group). Representative liver histology as viewed on a computer monitor is shown. Original magnification, ×100. Values are means ± S.E. of data obtained from the analysis of ob/ob mice and *adipo*(−/−)ob/ob mice. *, $p < 0.05$; **, $p < 0.01$. *E*, phosphorylation levels of AMPK in the muscle of ob/ob and *adipo*(−/−)ob/ob mice not treated (*open bars*) and treated (*filled bars*) with rimonabant after the clamp studies ($n = 5$/group). Results are representative of three independent experiments. Values are means ± S.E. pAMPK, phospho-AMPK; *n.s.*, not significant.

improvements induced by rimonabant were significantly less pronounced in the *adipo*(−/−)ob/ob mice, indicating that adiponectin is involved in the rimonabant-mediated amelioration of hepatic insulin resistance. In fact, although a significant decrease of the PEPCK expression levels was observed, the AMPK activity was significantly increased, and the hepatic TG content was decreased in the ob/ob mice; all of these changes were significantly less pronounced in the *adipo*(−/−)ob/ob mice lacking adiponectin. We reported from a previous study that adiponectin, especially HMW adiponectin, stimulates AMPK activation in the liver (26, 42). These findings suggest that rimonabant treatment activates AMPK in the liver via increasing the secretion of HMW adiponectin and then decreases the expression of PEPCK to inhibit glucose production and increase CPT-1 expression, thereby stimulating fatty acid oxidation in the liver.

On the other hand, rimonabant treatment also produced significant amelioration of hepatic insulin resistance in the absence of adiponectin. This amelioration was possibly attributable to the reduction of body weight (Fig. 1*A*) but not to suppression of MCP-1 and resistin expression (Fig. 2, *E* and *F*). Alternatively, this amelioration was possibly due to the direct activation of AMPK by rimonabant in the liver. In fact, recent reports have shown that AMPK activity was significantly higher in the liver of hepatocyte-specific CB-1 receptor knock-out mice, although the serum adiponectin levels in these animals remained unchanged (35, 43), suggesting that rimonabant treatment directly activates hepatic AMPK, even without the mediation of adiponectin, and decreases the expression of PEPCK to inhibit glucose production in the liver.

In addition, Osei-Hyiaman *et al.* (35) have reported that CPT-1 activity in the liver was significantly increased when systemic CB-1 receptors were blocked pharmacologically in wild-type mice. Moreover, hepatic CPT-1 activity increased, and hepatic TG content decreased when hepatic CB-1 receptors were blocked genetically (35). These data suggest that CB-1 receptor blockade stimulates CPT-1 activity and increases fatty acid combustion to decrease the TG content in the liver. Consistent with this, rimonabant actually increased CPT-1 expression and decreased the TG content in the livers of ob/ob and *adipo*(−/−)ob/ob mice. However, these effects were markedly attenuated in the *adipo*(−/−)ob/ob mice, suggesting that increased CPT-1 expression and decreased hepatic TG content by rimonabant were also mediated by adiponectin-dependent as well as adiponectin-independent pathways.

Based on our findings, we propose that there are two distinct pathways by which rimonabant ameliorates insulin resistance, one an adiponectin-dependent pathway and the other an adiponectin-independent pathway (Fig. 6). Rimonabant increases the plasma levels of adiponectin, in particular of HMW adiponectin, which induces AMPK activation and decreases gluconeogenesis in the liver, thereby ameliorating insulin resistance. On the other hand, in a manner independent of adiponectin, rimonabant directly induces AMPK activation and decreases gluconeogenesis in the liver, possibly via the hepatic CB-1 receptor (35, 43), which also contributes to ameliorating insulin resistance. In addition, rimonabant decreases food intake and increases energy expenditure, which are related to reduction of body weight. This body weight loss may be also associated with ameliorating insulin resistance via adiponectin-dependent and adiponectin-independent pathways (Fig. 6).

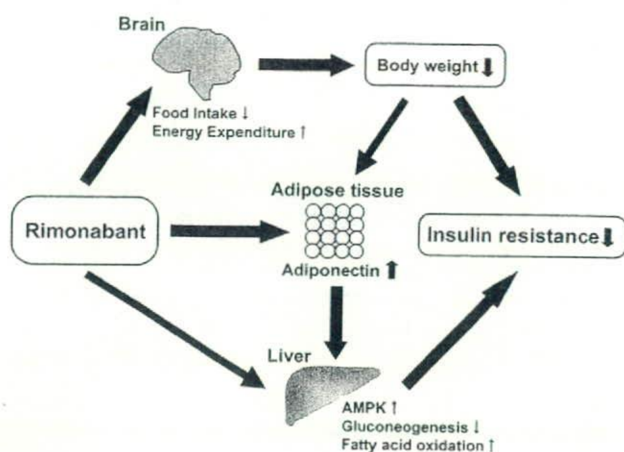

FIGURE 6. **Rimonabant ameliorates insulin resistance via both adiponectin-dependent and adiponectin-independent pathways.** There are two distinct pathways by which rimonabant ameliorates insulin resistance, one an adiponectin-dependent pathway and the other an adiponectin-independent pathway. Rimonabant increases the plasma levels of adiponectin, in particular of HMW adiponectin, which induces AMPK activation and decreases gluconeogenesis in the liver, thereby ameliorating insulin resistance. On the other hand, in a manner independent of adiponectin, rimonabant directly induces AMPK activation and decreases gluconeogenesis in the liver, possibly via hepatic CB-1 receptor, which also contributes to ameliorating insulin resistance. In addition, rimonabant decreases food intake and increases energy expenditure, which are related to reduction of body weight. This body weight loss may be also associated with ameliorating insulin resistance via adiponectin-dependent and adiponectin-independent pathways.

Rimonabant is metabolized in the liver by cytochrome P-450 CYP3A4 and amidohydrolase and excreted into the bile (44, 45). The oral bioavailability of rimonabant is low to moderate; this is due to the extensive first pass metabolism of the drug (European Medicines Agency). Therefore, in this study, the concentration in the liver of the orally administered rimonabant might be higher than that in other tissues, such as the muscle, because of the first pass effect of the liver. Although intraperitoneally administered rimonabant was reported in a previous study to significantly increase the glucose uptake in the soleus muscle of ob/ob mice (10), no improvement of the insulin resistance in the muscle was observed in our study. One of the reasons for this difference may be the lower concentration of rimonabant in the muscle due to the first pass effect of the liver.

In the four double-blind trials (RIO-Lipids (15), RIO-Europe (16), RIO-North America (17), and RIO-Diabetes (18)) the most frequent adverse events among individuals treated with rimonabant were nausea, dizziness, diarrhea, and insomnia, each occurring at a 1–9% greater frequency than that in the placebo group. In the RIO-Lipids, RIO-Europe, and RIO-North America, the drug had to be discontinued due to the development of psychiatric disorders (mainly depression) in 6–7% of rimonabant-treated individuals, an absolute increase of 2–5% over the frequency in the placebo group (44). Substance dependence with rimonabant has not been reported. The absence of the appearance of clinical signs in toxicology studies with a recovery period indicates that rimonabant does not possess the potential to produce withdrawal syndrome (European Medicines Agency).

Many reports have shown the efficacy of cannabinoid agonists in chronic pain (46). In a rodent model of inflammatory pain, anandamide, one of the endogenous cannabinoids, suppressed the development and maintenance of thermal hyperalgesia (47). This analgesic effect was diminished by concurrent administration of the CB-1 antagonist, rimonabant, and anandamide. Although rimonabant alters the sensitivity to pain (47), it does not necessarily induce pain itself. On the contrary, rimonabant has recently been shown to prevent indomethacin-induced intestinal injury by decreasing the levels of the proinflammatory cytokine, tumor necrosis factor α, in rodents (48), indicating its potential anti-inflammatory activity in acute and chronic diseases. In neurogenic inflammatory pain, including arthritis and neuropathy, many cytokines, especially tumor necrosis factor α, play a key role in the generation and maintenance of hyperalgesia (49). On the basis of these findings, Costa (50) indicated that the anti-tumor necrosis factor α effect of rimonabant might contribute to its anti-inflammatory activity and consequently to the relief of pain. However, further investigation and accumulation of further evidence on the effect of rimonabant on pain are needed. At least, in the four clinical trials mentioned above, side effects associated with pain, such as hyperalgesia or hypoalgesia, were not reported. Furthermore, it has been suggested that although females might perceive pain differently from males (51, 52), the anti-obesity effects of rimonabant appeared to be similar in males and females (European Medicines Agency).

In conclusion, this study demonstrated for the first time that rimonabant ameliorates insulin resistance via both adiponectin-dependent and adiponectin-independent pathways.

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Aberrant Crypt Foci as Precursors of the Dysplasia-Carcinoma Sequence in Patients with Ulcerative Colitis

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Abstract **Purpose:** Long-standing ulcerative colitis (UC) predisposes patients to the development of colorectal cancer, but surveillance of colitis-associated cancer by detecting the precancerous lesion dysplasia is often difficult because of its rare occurrence and normal-looking appearance. In sporadic colorectal cancer, aberrant crypt foci (ACF) have been reported by many investigators to be precursor lesions of the adenoma-carcinoma sequence. In the present study, we analyzed the genetic background of ACF to determine whether they could be precursors for dysplasia, and we examined the usefulness of endoscopic examination of ACF as a surrogate marker for surveillance of colitis-associated cancer.

Experimental Design: ACF were examined in 28 UC patients (19 patients with UC alone and 9 patients with UC and dysplasia; 2 of those patients with dysplasia also had cancer) using magnifying endoscopy. K-ras, APC, and p53 mutations were analyzed by two-step PCR RFLP, *in vitro*–synthesized protein assay, and single-strand conformation polymorphism, respectively. Methylation of p16 was analyzed by methylation-specific PCR.

Results: ACF that appeared distinct endoscopically and histologically were identified in 27 out of 28 UC patients. They were negative for K-ras, APC, and p53 mutations but were frequently positive for p16 methylation (8 of 11; 73%). In dysplasia, K-ras and APC mutations were negative but p53 mutation (3 of 5; 60%) and p16 methylation (3 of 5; 60%) were positive. There was a significant stepwise increase in the number of ACF from patients with UC alone to patients with dysplasia and to patients with cancer. Univariate and multivariate analyses showed significant correlations between ACF and dysplasia.

Conclusions: We have disclosed an ACF-dysplasia-cancer sequence in colitis-associated carcinogenesis similar to the ACF-adenoma-carcinoma sequence in sporadic colon carcinogenesis. This study suggests the use of ACF instead of dysplasia for the surveillance of colitis cancer and warrants further evaluation of ACF as a surveillance marker in large-scale studies.

It is commonly recognized that long-standing ulcerative colitis (UC) predisposes patients to the development of colorectal cancer (1). However, the detection of early colorectal cancer is often difficult in patients with UC because there is inflammation in the background mucosa and it predominantly represents flat-type ill-delineated lesions (2, 3). Therefore, colitis-associated cancer is often detected at an advanced stage and is characterized by a very poor prognosis. One approach to overcome this difficulty is to use dysplasia, which is considered to be a precancerous lesion in colitis-associated cancer, as a surrogate marker for early detection of colitis-associated cancer (4, 5). However, identification of dysplasia by endoscopy requires greater skill than detection of cancer itself because of its rare occurrence and apparently normal-looking appearance (6).

We previously succeeded in identifying aberrant crypt foci (ACF) in non-UC subjects using magnifying endoscopy (7) and showed that the number of ACF increased in the order of normal subjects, patients with adenomas and then patients with cancer, and proposed an ACF-adenoma-carcinoma sequence for sporadic colon carcinogenesis. Adler et al. also observed ACF using magnifying endoscopy and found that the number of rectal ACF in patients with colorectal cancer was significantly higher than in normal subjects (8). The increased number of ACF was further observed in patients with flat adenoma and cancer (9). Seike et al. showed that ACF could be a predictive factor for advanced rectal cancer by multivariate analysis (10). Thus, magnifying endoscopy has become a

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common methodology to observe ACF. Regarding the genetic abnormality of ACF, we found a highly frequent K-ras mutation and GSTP1-1 overexpression and also showed that GSTP1-1 endows ACF with resistance to bile salt–induced apoptosis (11–13).

With regard to the genetic abnormality of colitis-associated cancer and dysplasia, mutations of *K-ras* and *APC* are relatively rare (14–17), and in contrast, *p53* mutation is frequently positive (16, 18, 19). Moreover, hypermethylation of genes such as the *p16* gene has recently been detected (20, 21). On the basis of these previous reports, we first attempted to define ACF as precursors for colitis-associated dysplasia by analyzing their genetic background, including mutation of *K-ras, APC, p53,* and hypermethylation of p16, and then examined the feasibility of using ACF as surrogate markers for the surveillance of colitis-associated cancer.

Materials and Methods

Subjects. This study was approved by the ethics committee of Sapporo Medical University. Fifty-six subjects were enrolled after obtaining written informed consent. The subjects were comprised of 28 UC patients (19 patients with UC alone and 9 patients with UC and dysplasia; 2 of those patients with dysplasia also had cancer), 24 healthy subjects, and 4 patients with Crohn's disease as a control. Average ages and male/female ratios in these groups were as follows: UC, 38.3 ± 6.7 years and 1/1; healthy subjects, 41.5 ± 7.8 years and 3/5; Crohn's disease patients, 30.3 ± 7.4 years and 2/2. The diagnosis of UC was made according to established criteria, including clinical symptoms, radiological findings, blood examination, endoscopy, and pathologic observation of inflamed intestinal mucosa.

Magnifying endoscopy. UC patients in remission underwent magnifying endoscopy, which was done as described previously (7, 11). In order to improve the visualization of ACF (i.e., accurate evaluation of ACF number), plenty of polyethyleneglycol was administered before examination. All patients underwent total colonoscopy. After observation of the entire colorectum, the lower rectum from the middle Houston valve to the dentate line was washed with plenty of water, stained with 0.2% methylene blue, and again washed with sufficient water for identification of ACF. Biopsies were taken under magnifying endoscopy as described previously (7, 11). In this particular study, to avoid laborious and lengthy procedures considering the future application of ACF as a surveillance marker, the observation of ACF was limited to the lower rectum on the basis of our previous report; the number of ACF in the lower rectum correlated with that in entire colorectum (7). All procedures were recorded on videotape and evaluated by two independent observers who were unaware of the subjects' clinical histories.

ACF criteria. ACF were defined as lesions in which crypts were more darkly stained with methylene blue than normal ones and had larger diameters, often with oval or slit-like lumens and thicker epithelial linings (22, 23).

Two-step PCR and RFLP for detection of K-ras codon 12 mutations. Cellular DNA was extracted from the biopsy specimens and used as a template for PCR. The PCR products were amplified using mismatched primers and analyzed by RFLP to detect point mutations in codon 12 of the *K-ras* gene, as described previously (12, 24).

In vitro*–synthesized protein assay for detection of mutations in APC.* *In vitro*–synthesized protein assays were performed according to a method described previously (25). In brief, primer pairs were prepared for segments 3 (codons 686-1022) and 4 (codons 996-1693) of the *APC* gene, which include the whole mutation cluster region. These primer pairs were specially designed to place the necessary transcriptional and translational regulatory sequences at the 5′-ends of the PCR products. Genomic DNA were extracted from ACF and dysplasia tissue samples were obtained by microdissection. After amplification of the *APC* gene, the PCR products were used directly, without cloning, as templates in coupled transcription and translation reactions (Promega Corp.) in a mixture containing 10 μCi of ^{35}S-methionine. The proteins thus synthesized were analyzed on 10% to 20% gradient SDS polyacrylamide gels and visualized by autoradiography.

Single-strand conformation polymorphism analysis of p53. Four primer pairs for exons 5, 6, 7, and 8/9 (Takara), which include the hotspot region of *p53* mutation were used. Single-strand conformation polymorphism analyses were done according to the method described previously (26). In brief, genomic DNA were amplified using each primer pair. Aliquots of the PCR product were denatured for 5 min at 80°C in a sample buffer containing 98% formamide, and then cooled quickly on ice. Each sample was electrophoresed on a 15% polyacrylamide gel, which was stained using a silver staining kit (Bio-Rad).

Analysis of hypermethylation of p16. Bisulfite treatment of DNA was done as described previously (27). Briefly, 2 μg of genomic DNA were denatured in 0.2 mol/L of NaOH at 37°C for 20 min, followed by incubation with 3 mol/L of sodium bisulfite (Sigma Chemical Co.); hydroquinone (Sigma Chemical Co.) was added at a final concentration of 0.5 mmol/L. The reaction was done at 55°C for 16 h. After treatment, modified DNA was purified using a Wizard DNA Clean-Up kit (Promega) as recommended by the manufacturer, and resuspended in 30 μL of distilled water. Two microliters of the bisulfite-incubated DNA were used as a template for each bisulfite-PCR, and primer pairs were used as described previously (27). After amplification, each PCR sample was electrophoresed on 10% to 20% polyacrylamide gels, stained with ethidium bromide and directly visualized under UV illumination.

RNA extraction and reverse transcription-PCR. Total RNA was isolated from the frozen samples of ACF and normal mucosa of UC patients using the total RNA isolation system (Promega). Reverse transcription-PCR (RT-PCR) was done as previously described (28). Briefly, the reverse transcription reactions were achieved using avian myeloblastosis virus reverse transriptases. Then, the p16^{INK4A}-specific exon 1 was amplified with primers 5′-ATGGAGCCTTCGGCTGACTGG-3′ and 5′-GATCGGCCTCCGACCGTAAC-3′. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard.

Statistical analysis. The number of ACF in relation to potential risk factors for colitis-associated cancer were compared by Mann-Whitney *U* test. Multivariate analysis was carried out by multiple logistic regression analysis using SAS software (SAS Institute Japan).

Results

Endoscopic appearance and histology of ACF in UC patients. An endoscopic view of dysplasia, which is often difficult to identify by standard endoscopy in a patient with UC is shown in Fig. 1A. Histologically, the nuclei of goblet cells are hyperchromatic, have lost their normal polarity, and show some nuclear stratification. These characteristics are consistent with low-grade dysplasia (29). Figure 1D shows a representative endoscopic view of ACF in a patient with UC (colitis ACF) in comparison with typical ACF in non-UC patients (non-UC ACF), which we reported previously (Fig. 1G). Both types of ACF could be identified as a focus consisting of large crypts darkly stained with methylene blue. However, in comparison to the typical non-UC ACF, the lining of each crypt of the colitis ACF was obscure and the boundaries of individual crypts were unclear. Moreover, most of the colitis ACF showed distorted shapes in contrast to round or oval shapes of the non-UC ACF in the majority. Histologically, the

colitis ACF specimens showed marked infiltration of lymphocytes in the stroma (Fig. 1E) as compared with non-UC ACF (Fig. 1H). They also showed a more diverse range of crypt sizes, enlarged nuclei in epithelial cells, and increased chromatin staining.

We identified a total of 164 ACF in 27 out of 28 patients with UC by using methylene blue in magnifying endoscopy, and no side effects were noted. Of these, 147 (91.3%) showed the typical appearance of colitic ACF as illustrated in Fig. 1D. Only 17 ACF (9.7%) in these patients showed the typical appearance of non-UC ACF. Therefore, we confined further examinations to the colitis ACF.

Colitis-associated colorectal cancer is reported to have a higher incidence of histologically mucinous type tumors than sporadic colorectal cancer. It has also been reported that goblet cell hyperplasia is frequently observed in dysplasia from patients with UC and that the frequency of goblet cell hyperplasia is positively correlated with the duration of UC (30). Therefore, goblet cells in nine colitis ACF, nine non-UC ACF, seven dysplasia, and seven normal rectal epithelial specimens were examined by Alcian blue staining. The frequencies of goblet cells observed in colitis ACF and dysplasia tissues were 49.5 ± 9.7% and 52.7 ± 14.2%, respectively. They were significantly greater than those in the normal background mucosa of ACF tissues (32.3 ± 5.9%) and in non-UC ACF (35.7 ± 15.4%), suggesting the existence of goblet cell hyperplasia in colitis ACF as well as dysplasia. These results are also consistent with the hypothesis that ACF are precursor lesions of dysplasia in patients with UC.

Analysis of K-ras mutation in colitis ACF and dysplasia. Because *K-ras* mutations have frequently been detected in ACF from non-UC patients (11, 31, 32), we screened colitis ACF specimens and dysplasia tissue from UC patients for *K-ras* codon 12 mutations by a two-step PCR-RFLP method. *K-ras* mutations were found in 20% (2 of 10) and 0% (0 of 5) of colitis ACF and dysplasia specimens, respectively, from UC

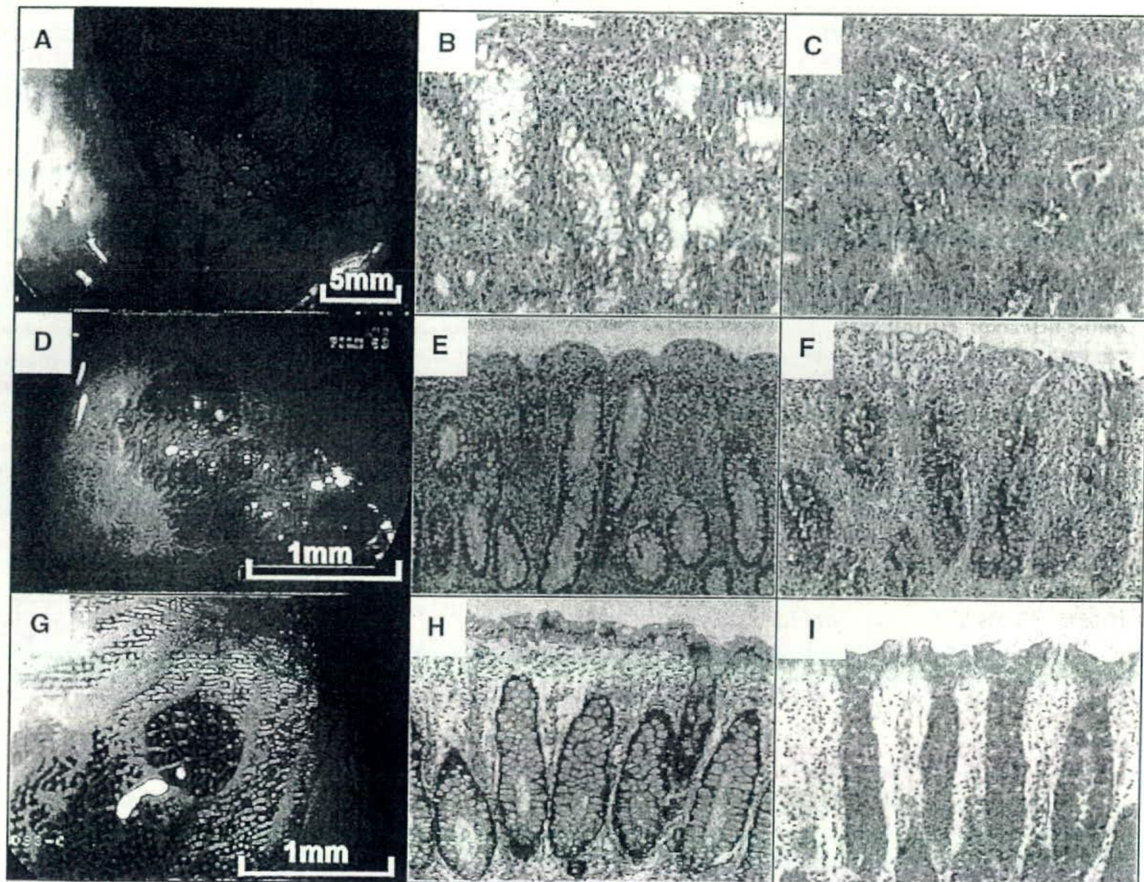

Fig. 1. Endoscopic and histologic features of dysplasia (*A-C*), ACF in a patient with UC (*D-F*), and ACF in a non-UC patient (*G-I*). *A*, dysplasia in a UC patient, which was not visible unless chromoendoscopy was done. *B*, the crypt was lined by columnar epithelia with some nuclear stratification, hyperchromatic nuclei, and loss of normal polarity (H&E; magnification, ×150). *C*, increased numbers of goblet cells were seen in the dysplasia of UC patients. Some dystrophic goblet cells were observed (Alcian blue; magnification, ×150). *D*, a representative ACF in a UC patient, which was characterized by darker staining with methylene blue and larger crypts with thicker epithelial lining than the background mucosa. The lining of each crypt was obscure and the boundaries of individual crypts were more unclear than in non-UC ACF (*G*). *E*, the colitis ACF showed marked infiltration of lymphocytes in the stroma, a more diverse range of crypt sizes, enlarged nuclei in epithelial cells, and increased chromatin staining compared with non-UC ACF (H&E; magnification, ×120). *F*, an increase in the number of goblet cells was seen in colitis ACF, similar to dysplasia in UC patients. Some dystrophic goblet cells were also identified (Alcian blue; magnification, ×120). *G*, a representative non-UC ACF consisting of crypts with round and oval lumens and with a wide pericryptal space. *H*, non-UC ACF showed slight enlargement, irregularity, and elongation of the ducts (H&E; magnification, ×150). *I*, the number of goblet cells in non-UC ACF was apparently fewer than that in colitis ACF (Alcian blue; magnification, ×150).

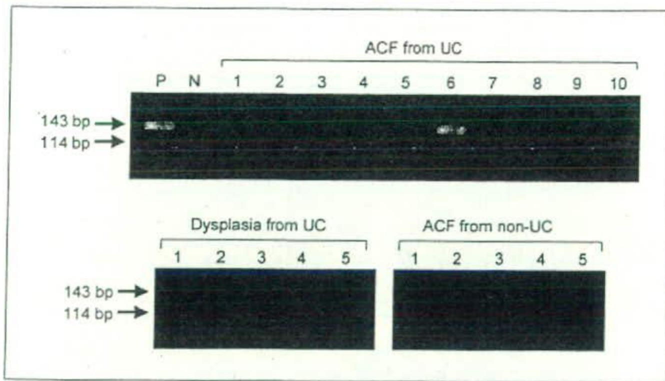

Fig. 2. Analysis for *K-ras* mutation in colitis ACF, dysplasia from UC patients, and non-UC ACF by two-step PCR and RFLP. A pancreatic cancer cell line, APSC (ATCC CRL1862; American Tissue Culture Collection), which is known to have a K-ras point mutation, was used as a positive control (*P*). A normal colonic mucosa was used as a negative control. *K-ras* mutations were found in 20% (2 of 10) and 0% (0 of 5), respectively, of colitis ACF and dysplasia from UC patients. In contrast, mutations were detected in four of five patients with (80%) non-UC ACF.

patients. In contrast, mutations were detected in 4 of 5 (80%) non-UC ACF specimens (Fig. 2), consistent with our previous reports and those of other laboratories (7, 11, 14, 15). Thus, the frequency of *K-ras* mutations in colitis ACF was relatively low compared with that of non-UC ACF.

Analysis of APC mutation in colitis ACF and dysplasia. Because *APC* mutation is an early genetic event in colorectal carcinogenesis in non-UC patients (32-34), we examined *APC* mutations in colitis ACF and dysplasia specimens. Segments 3 and 4 of the *APC* gene, which include the entire mutation cluster region, were analyzed by an *in vitro* - synthesized protein assay in 11 colitis ACF and 2 dysplasia tissue specimens. No *APC* mutations were detected in any of the 11 colitis ACF (0 of 11, 0%) or the 2 dysplasia specimens (0 of 2, 0%; Fig. 3). Likewise, no *APC* mutations were detected in any of the 7 ACF specimens from non-UC patients (data not shown), consistent with our previous report (11).

Analysis of p53 mutation in colitis ACF and dysplasia. It has been reported that p53 mutations are frequently detected in dysplasia and cancer tissues from patients with UC (16, 18, 19). Therefore, we investigated for *p53* mutations in the hotspot region (exons 5-9) employing nonradioisotopic single-strand conformation polymorphism in 11 colitis ACF and 5 dysplasia specimens from patients with UC. No mutations were detected in any of the exons 5, 6, 7, or 8/9 in the 11 ACF specimens, whereas mutations were detected in exons 5, 6, or 8/9 of the 5 dysplasia specimens (Fig. 4). Overall, for exons 5 to 9, *p53* mutations were found in 3 of the 5 dysplasia specimens (60%) but in none of the 11 ACF lesions (0%) from patients with UC.

Hypermethylation of the p16 *gene promoter in colitis ACF and dysplasia.* Recently, it was reported that the *p16* gene promoter is often hypermethylated in dysplasia and cancer from UC patients (20, 21). Therefore, we examined the methylation status of the promoter region of the *p16* gene in 11 ACF, 5 dysplasia, and 4 normal epithelia specimens from 4 UC patients using methylation-specific PCR. No methylation of the *p16* gene promoter, which was represented by a 152 bp band, was detected in any of the 4 normal epithelia specimens. However, it was found in 8 of the 11 ACF (73%) and in 3 of the 5 dysplasia specimens (60%; Fig. 5A).

We then determined the expression of $p16^{INK4A}$ mRNA employing RT-PCR in eight ACF, two dysplasia, and four normal epithelia specimens from the other four UC patient groups. The reason we dealt with specimens from other UC groups (four patients) than the group (four patients) for methylation analyses, was that analyses of methylation and mRNA on the same small specimens was technically difficult. Nevertheless, the $p16^{INK4A}$ mRNA was readily detected in all four specimens of normal epithelia. Although it was detectable in only two of eight (25%), very faintly detectable in one of eight (12.5%), and undetectable in five of eight (63%) ACF specimens and was undetectable in two of two dysplasia specimens (Fig. 5B). These results suggested that $p16^{INK4A}$ expression is suppressed by the methylation of its promoter.

The number of colitis ACF in UC patients with or without dysplasia. If ACF are indeed precursor lesions of dysplasia, it would be expected that UC patients with dysplasia would have more ACF than those without dysplasia. Therefore, we investigated the number of ACF in UC patients with and without dysplasia and compared them. The number of ACF in the dysplasia-positive group (8.7 ± 4.5) was significantly higher than that in the dysplasia-negative group (3.5 ± 2.6; $P = 0.0112$). In particular, the number of ACF in the two patients with both dysplasia and cancer were 17 and 13, respectively, which represents very high numbers even for the dysplasia-positive group. All cases in the dysplasia-positive

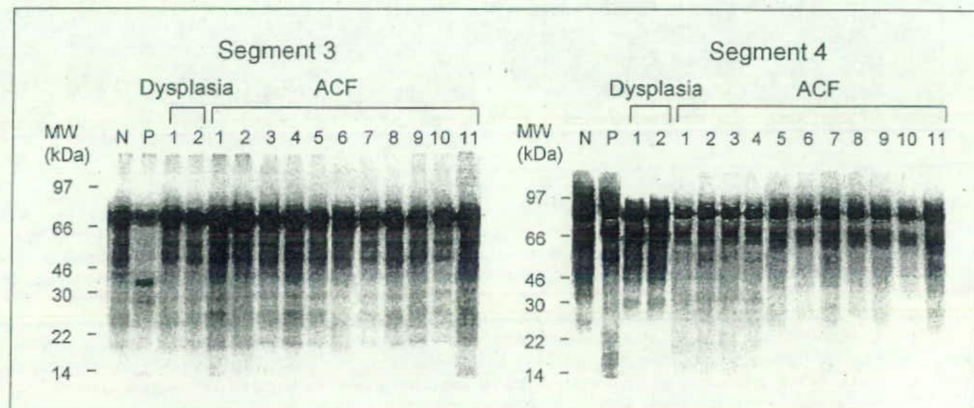

Fig. 3. Analysis for *APC* mutations in colitis ACF, dysplasia from UC patients, and non-UC ACF by *in vitro* - synthesized protein assay. Segments 3 and 4 of the *APC* gene, which include the whole mutation cluster region, were analyzed. A colonic adenoma was used as a positive control and normal colonic mucosa was used as a negative control. No *APC* mutations were detected in any of the 11 colitis ACF samples (0 of 11, 0%) or in any of the two dysplasia specimens (0 of 2, 0%).

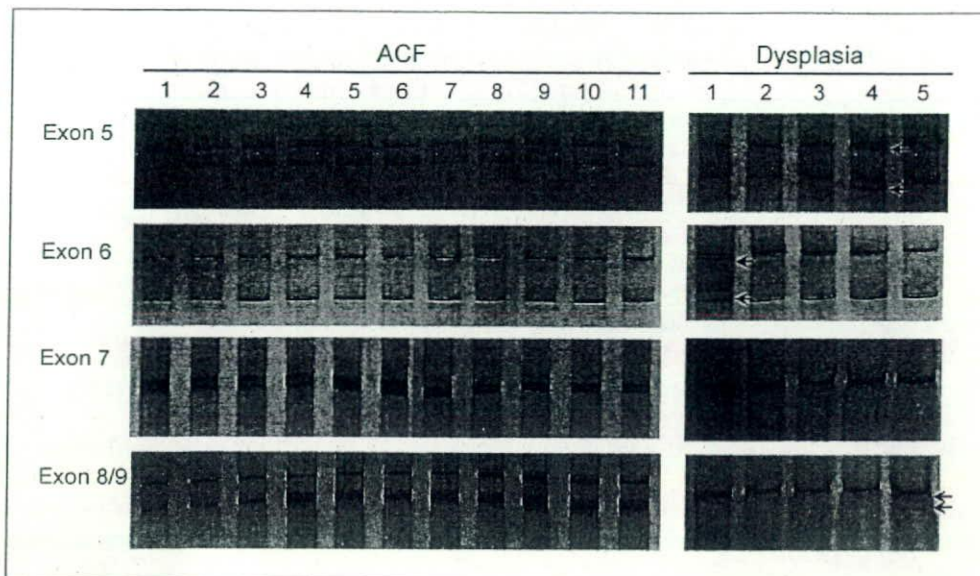

Fig. 4. Analysis for *p53* mutations in colitis ACF and dysplasia from UC patients by nonradioisotopic single-strand conformation polymorphism assay. The hotspot regions (exons 5-9) of p53 mutations were analyzed. No mutations were detected in any of the exons 5, 6, 7, or 8/9 in the 11 ACF specimens (0 of 11, 0%), whereas mutations were detected in exons 5, 6, or 8/9 of the dysplasia specimens (3 of 5, 60%).

group had five or more ACF. The mean number of ACF in age-matched healthy subjects (1.0 ± 1.7) was apparently smaller than that in either the dysplasia-positive or dysplasia-negative group of UC patients. The mean number of ACF in the four patients with Crohn's disease, an inflammatory bowel disease from which cancer develops at a low rate, was only 0.3 ± 0.6 (Fig. 6).

The number of colitis ACF in relation to potential risk factors for colitis cancer. We analyzed the relationship between the number of ACF and potential risk factors for colitis cancer such as gender, age at onset, the extent of lesions, duration of disease, and the existence of dysplasia. Univariate analyses showed significant correlations between ACF numbers and the existence of dysplasia ($P = 0.0112$) or duration of disease ($P = 0.0306$). There were no significant correlations between the number of ACF and gender, age, or extent of lesions (Table 1). Multiple logistic regression analysis of the relationship between dysplasia and various background factors showed significant correlations between the presence of dysplasia and the number of ACF ($P = 0.0189$) or disease duration ($P = 0.0492$; Table 2).

Discussion

In this study, we successfully and to our knowledge, for the first time, identified colitis ACF employing magnifying

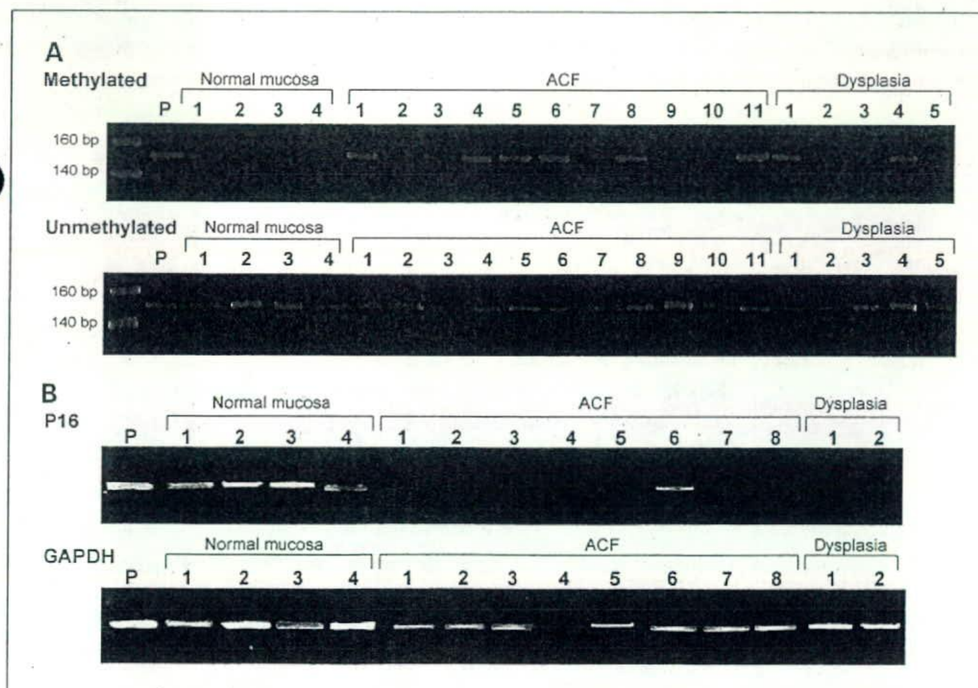

Fig. 5. *A*, analysis for *p16* methylation in colitis ACF and dysplasia specimens from UC patients by methylation-specific PCR. A colonic cancer specimen from a non-UC patient was used as a positive control. Methylation of the *p16* gene promoter, which was represented by a 152 bp band, was found in 8 of the 11 colitis ACF specimens (73%) and in 3 of the 5 dysplasia specimens (60%). *B*, expression of $p16^{INK4A}$ mRNA in colitis ACF and dysplasia specimens from UC patients analyzed by RT-PCR. A colonic cancer specimen from a non-UC patient was used as a positive control. $p16^{INK4A}$ mRNA was clearly detected in only 2 of 8 (25%), very faintly detected in 1 of 8 (12.5%), and was not detected in 5 of 8 (63%) ACF specimens. It was absent in two of the dysplasia specimens.

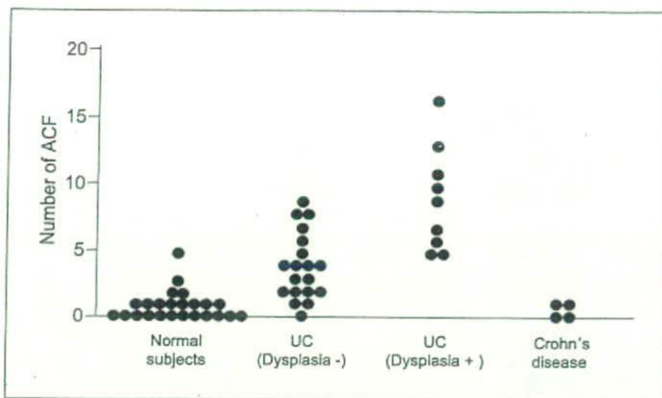

Fig. 6. The number of ACF in healthy volunteers and patients with UC or Crohn's disease. The number of ACF differed significantly ($P = 0.0112$) between the dysplasia-positive group (8.7 ± 4.5) and the dysplasia-negative group (3.5 ± 2.6). The mean number of ACF in the four cases of Crohn's disease was only 0.3 ± 0.6.

endoscopy, which were more darkly stained with methylene blue than normal crypts and had larger diameters with oval or slit-like lumens and thicker epithelial linings (7, 11). The appearance of colitis ACF was distinct from sporadic ACF. Endoscopically, the boundaries of individual crypts in colitis ACF were obscure in contrast to the clear lining of each crypt in sporadic ACF, and most of the colitis ACF showed distorted shapes in contrast to round or oval shapes of sporadic ACF. Histologically, much more lymphocyte infiltration was seen in colitis ACF than in sporadic ACF.

Differences were also evident with respect to genetic background. Sporadic ACF were frequently positive for *K-ras* mutation and p16 overexpression, as previously reported by us and others (11, 12, 28, 35), whereas colitis ACF were essentially negative for *K-ras* mutation and also negative for p16 expression due to hypermethylation of the gene. These results suggest that the etiology of ACF may be different in UC patients from that in sporadic ACF subjects. In this context, it is intriguing that in other types of inflammation-associated carcinogenesis, such as hepatitis C–associated hepatocellular carcinoma and chronic gastritis-associated gastric cancer, silencing of the *p16* gene (p16 hypermethylation) is frequently observed (36–38).

With regard to the relationship between colitis ACF and dysplasia, it is highly plausible that the former are precursor lesions of the latter because the gene abnormalities of both lesions were similar in terms of negativity for *APC* and *K-ras* and positivity for *p16* hypermethylation. Multivariate analysis showed a close correlation between the number of ACF and occurrence of dysplasia, which also strongly supported the precursor theory of ACF. The close correlation between lesions and the fact that ACF were readily detectable in higher numbers than dysplasia, which requires total chromoendoscopy spraying methylene blue on the entire colorectum for detection, suggests that ACF are a more appropriate surveillance marker than dysplasia for colitis-associated cancer.

Incidentally, the ACF found in UC patients were not all colitis ACF but were mixed with typical sporadic (non-UC) ACF, as far as endoscopic appearance was concerned. However, the incidence was very low (only 9.7%). This may be explained by the fact that the prevalence of sporadic ACF sharply increases after the age of 40 to 50 years (7), whereas the mean age of UC patients in this study was 38.3 ± 6.7 years. Nevertheless, because of the low incidence, sporadic ACF contamination with colitis ACF would not hamper the usefulness of colitis ACF as a surveillance marker.

A possible obstacle to using colitis ACF as a surveillance marker is that patients are obliged to undergo endoscopic examination, which itself may aggravate UC activity. However, this is unlikely to be a significant obstacle as long as endoscopy is done only when UC is in an inactive state and the survey of ACF is limited to the rectum, spending only 10 to 15 min on the whole procedure on the basis of our previous finding that the number of ACF in the rectum correlated with that in the entire colorectum (7). In this study, indeed, no particular aggravation of UC activity was observed in any of the patients with the evidential results of univariate and multivariate analyses supporting the validity of the use of rectal ACF in place of entire colorectal ACF as a surveillance marker.

In conclusion, in this study, we disclosed an ACF-dysplasia-cancer sequence in colitis-associated carcinogenesis similar to the ACF-adenoma-carcinoma sequence in sporadic colon carcinogenesis. We then proposed the feasibility of using ACF instead of dysplasia for the surveillance of colitis-associated cancer. Further evaluation of ACF as a surveillance marker in large-scale studies is warranted.

Table 1. Univariate association of ACF with potential risk factors for colorectal cancer in UC patients

Risk factor		Number of ACF-like lesions	*P*
Gender	Male	5.0 ± 3.9	0.3166
	Female	6.7 ± 4.9	
Age at onset (y)	<40	5.7 ± 4.7	0.8830
	R40	6.0 ± 4.3	
Extension	Total colon	7.0 ± 4.6	0.1179
	Left side colon	4.3 ± 3.8	
Duration (y)	<8	3.6 ± 2.2	0.0306
	R8	7.3 ± 4.9	
Dysplasia	Positive	8.7 ± 4.5	0.0112
	Negative	3.5 ± 2.6	

Acknowledgments

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Table 2. Multiple logistic regression analysis of risk factors associated with dysplasia in patients with UC

Risk factor	Odds ratio (95% confidence interval)	*P*
Gender	3.274 (0.709-25.195)	0.1645
Age	0.955 (0.801-1.085)	0.5138
Extension	1.424 (0.649-3.347)	0.1793
Duration	1.398 (0.785-1.603)	0.0492
ACF	1.533 (1.073-2.191)	0.0189

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REVIEW

Chemoprevention of colorectal cancer -experimental and clinical aspects-

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Abstract : Colorectal cancer is a leading cause of cancer-related mortality worldwide. Therefore, an appropriate prevention strategy should be urgently established. Chemoprevention involves the use of oral agents to suppress the development of cancer. Recent progress in the molecular analysis of colorectal cancer has revealed many candidate molecules for chemoprevention. Many new agents targeting these molecules have also been developed. These agents are largely classified into three categories : 1) Signal transduction modulators including epidermal growth factor (EGF) receptor inhibitors, anti-vascular endothelial growth factor (VEGF) antibodies, and inhibitors of oncogene products. 2) Epigenetic modulators including peroxisome proliferative activated receptor (PPAR)-γ agonists, estrogen receptor (ER)-β, and histone deacetylase inhibitors. 3) Anti-inflammatory modulators including cyclooxygenase (COX)-2, EP 1-4, and NF-kB. Of these agents, some actually proceeded to human clinical trials, and have been shown to be active chemopreventive agents. J. Med. Invest. 56 : 1-5, February, 2009

Keywords **:** *colorectal cancer, chemoprevention, aberrant crypt foci*

INTRODUCTION

Colorectal cancer is a disease with a high incidence and mortality rate, and has been increasing in prevalence worldwide (1). Therefore, various prevention strategies have been investigated. Primary prevention attempts to prevent the occurrence of colorectal cancer by lifestyle modification, and secondary prevention aims to arrest the progression of colorectal cancer through early diagnosis and treatment. In addition to these, recently, chemoprevention, the use of oral drugs to prevent cancer, has attracted much attention. Many compounds have been tested to assess their inhibition of colorectal carcinogenesis in animal models, and some of them have been proceeded to clinical trials for chemoprevention.

Recent progress in the molecular analysis of colorectal carcinogenesis has revealed many candidate molecules for chemopreventive agents. In this review, we summarize new findings regarding experimental data and clinical trials for the chemoprevention of colorectal cancer.

ANIMAL MODEL OF COLORECTAL CANCER

It is very important to use an animal model for the evaluation of chemopreventive agents against colorectal carcinogenesis. There are two kinds of rodent model for colorectal cancer. One is the model of chemical carcinogenesis employing carcinogens such as azoxymethane, 1, 2-dimethylhydrazine (DMH), N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG), etc. Of these, the azoxymethane model is

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the most widely used as a model of sporadic colorectal carcinogenesis, and is reportedly very similar to human colorectal cancer in terms of the clinical symptoms, clinical course, and pathological findings (2). The other one is the genetic model harboring gene mutations such as APC, p53, etc. The Min mouse and Apc delta716 knockout mouse, both of which have APC mutations, are also used worldwide (3, 4).

In 1987, Bird reported a tiny lesion consisting of large, thick crypts in a methylene blue-stained specimen of the colon from mice treated with azoxymethane, and suggested to be a precursor lesion of colorectal cancer in the animal model (5). Then, abundant evidence was reported to support that aberrant crypt foci (ACF) are a precursor lesion of colorectal cancer. Thus, ACF are often used as a target lesion to test chemopreventive effects in animal models of colorectal carcinogenesis.

CHEMOPREVENTIVE AGENTS AND TARGET MOLECULES

Recent progress in the molecular analysis of colorectal cancer has made it possible to target a specific molecule for chemoprevention (6). Many promising target molecules have been reported so far (Table 1). These can be mainly classified into 3 categories based on the mechanism : 1) signal transduction modulation, 2) epigenetic modulation, and 3) anti-inflammatory modulation.

1) Signal transduction modulator

The signal transduction pathway has been searched for a long time as a target of chemotherapy and chemoprevention. EGF receptor inhibitors (Erlotinib, etc.), anti-EGF receptor antibody (Cetuximab), and anti-VEGF antibody (Bevacizumab) are well-known as therapeutic agents for cancer and commonly used worldwide (7). Although these agents have not yet been applied to chemoprevention, they themselves or their analogues may be put to practical use as chemopreventive agents of colorectal cancer in the future. Since mutations of K-ras and p53 are frequently observed in colorectal cancer, their oncogenic pathway is a possible target. Anti-ras agents such as Tipifarnib and perilyl alcohol, and anti-p53 agents such as CP31398 have been reported to inhibit colorectal carcinogenesis in animal models (8). Other signal transduction modulators targeting Bcl-2, ODC, GST-pi, etc., have also been examined for their chemopreventive effect on colorectal cancer.

2) Epigenetic modulation

It is well known that peroxisome proliferator-activated receptor (PPAR)-γ and -δ play a role in the

Table 1 Candidate of chemopreventive agents and target molecules for colorectal cancer

Mechanism	Target	Agents
Signal transduction modulation	EGF receptor	Cetuximab, Erlotinib
	Bcl-2	ABT-737
	Ras	Tipifarnib, Perillyl alcohol
	p53	CP31398
	Matrixmetalloproteinases	Marimistat, Prinomastat
	ODC	DFMO, NSAIDs, Retinoids
	VEGF/VEGF receptor	Bevacizumab
	GST-pi	HGBP, TLK119
Epigenetic modulation	Peroxisome proliferator activated receptor (PPAR)	Rosiglitazone, Pioglitazone
	Vitamin D	Vitamin D3 analogue
	ER-β	Resveratorol, TAS-108
	Histone deacetylase	SAHA
	Retinoic acid receptor	Retinoids
Anti-inflammation	COX-2	NSAIDs, Celecoxib, Etodorac
	EP1-4	ONO-8711
	NF-κB	Bortezomib, Curcumin, Tea polyphenols, Statins, NSAIDs

process of colorectal carcinogensis. Of these, PPAR-γ agonists such as rosiglitazone and pioglitazone reportedly inhibit the formation of colorectal cancer in animal models (9). Currently, they are being tested in human trials. There are some studies in which vitamin D inhibited the development of colorectal adenoma and cancer. Other epigenetic modulators including ER-β, histone deacetylase, and retinoic acid receptor have been reported to be potential chemopreventive agents in animal models.

3) Anti-inflammatory modulation

Cyclooxygenase-2 (COX-2) is reportedly overexpressed in colorectal adenoma and cancer of rodents and humans. It is also reported that COX-2 promotes the cell growth and inhibits apoptosis of colorectal epithelia. When an Apc delta716 knockout mouse, a model of human familial adenomatous polyposis, was crossed with a COX-2 knockout mouse, the number and size of intestinal polyps were markedly reduced (10). Moreover, there are many studies showing that selective COX-2 inhibitors suppressed colorectal adenoma and cancer. Thus, the efficacy of targeting the COX-2 molecule for chemoprevention was theoretically confirmed in animal models. There are also many other anti-inflammatory agents including EP1-4 and NF-κB currently under investigation.

CLINICAL TRIAL FOR CHEMOPREVENTION

Representative human chemopreventive trials are shown in Table 2. They are mainly classified into 3 categories according to the target lesion. The first one is a trial that targets a pre-existing polyp. Giardiello, *et al.* reported that sulindac significantly suppressed the number and size of polyps in familial adenomatous polyposis patients in 1993 (11). This study prompted investigators to conduct a trial to examine whether or not sulindac suppresses sporadic polyps. However, it did not significantly suppress the number or size of the polyps (12). This trial revealed that a pre-existing polyp is not necessarily an appropriate target for chemoprevention; a large polyp close to a cancer may not be able to respond to chemopreventive agents. Thus, chemoprevention targeting the development of a new polyp in polypectomized patients was conducted thereafter. Several randomized trials showed that aspirin inhibited the development of polyps. Since COX-2 was shown to be a good target molecule for chemoprevention in animal experiments, as noted above, two large-scale randomized clinical trials using a selective COX-2 selective inhibitor (celecoxib) were performed. Arber, *et al.* reported that celecoxib (400 and 800 mg/day) significantly reduced the new development of

Table 2 Representative chemopreventive studies for colorectal cancer

	Sporadic/FAP	Agents	Period	Results	Author
Pre-existing polyp					
	FAP	Sulindac	4 yr	No change	Giardiello, *et al.* (2002)
	FAP	Celecoxib	6 mo	30% reduction	Steinbach, *et al.* (2000)
	Sporadic	Sulindac	4 mo	No change	Ladenheim, *et al.* (1995)
	FAP	Sulindac	9 mo	65% reduction	Giardiello, *et al.* (1993)
Development of new polyp					
	Sporadic	Celecoxib	3 yr	38% reduction	Bertagnolli, *et al.* (2006)
	Sporadic	Celecoxib	3 yr	35% reduction	Arber, *et al.* (2006)
	Sporadic	Aspirin	1 yr	37% reduction	Sandler, *et al.* (2003)
	Sporadic	Aspirin	1~3 yr	17% reduction	Baron, *et al.* (2003)
	Sporadic	Calcium	4 yr	15% reduction	Baron, *et al.* (1999)
Development of cancer					
	Sporadic	Vitamin D Calcium	6 yr	32% reduction No change	Martinez, *et al.* (1996)
	Sporadic	Vitamin D Calcium	4 yr	26% reduction No change	Bostick, *et al.* (1993)
	Sporadic	Folic acid	6 yr	31% reduction	Giovannucci, *et al.* (1993)

adenoma compared to a placebo group (13). Bertagnolli, *et al.* also reported that celecoxib (400 and 800 mg/day) significantly reduced the development of adenoma in a different large-scale trial (14). However, in these trials, severe cardiovascular events including myocardial infarction and stroke occurred in about 20% of cases. Therefore, it is considered that the COX-2 inhibitor is an effective agent for the prevention of colorectal cancer, but it cannot be recommended for chemoprevention because of potential cardiovascular events.

The third one is a trial that targets the development of cancer. This kind of trial is theoretically ideal because it examines if each agent indeed suppresses the development of cancer itself. However, it takes more than 4 years, and prolongation of the trial sometimes causes severe side effects and poor compliance.

CHEMOPREVENTION TARGETING ACF

Since ACF are the earliest precursor lesions of colorectal cancer (15, 16), they would be an appropriate target for chemoprevention (Fig. 1). The advantages of using ACF as targets over a polyp and cancer are as follows : (1) short-term treatment for evaluation, (2) fewer complications caused by drugs, and (3) good compliance. Thus, we performed an open trial in which sulindac was administered for various periods to subjects positive for ACF. The results showed that the majority of ACF were eradicated after only a few months. Based on this, we next performed a randomized double-blind trial targeting ACF consisting of groups receiving sulindac, etodolac (a selective COX-2 inhibitor), or a placebo. The detailed results of this study will be clarified in the near future.

EPILOGUE

Many candidate agents for chemoprevention are currently being tested, and some of them have actually shown potential chemopreventive activity in human trials. Although the COX-2 inhibitor failed to be a major chemopreventive agent, other effective new agents will be identified in the near future.

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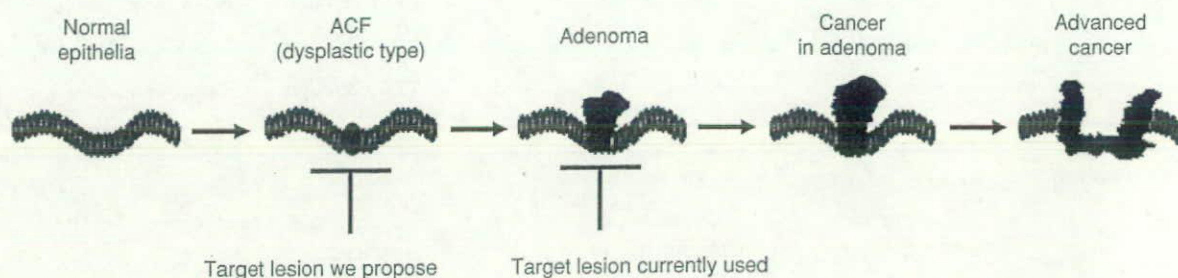

Figure 1 Colorectal carcinogenesis and target lesions for chemoprevention. In the majority of chemopreventive studies performed so far, adenoma has been used as a target lesion for evaluation. We propose the use of aberrant crypt foci (ACF), an earlier lesion, as a target. This makes it possible to evaluate the effect of a chemoprevenitve agent within a shorter period.

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Resolution of liver cirrhosis using vitamin A–coupled liposomes to deliver siRNA against a collagen-specific chaperone

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