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Bi-directional Regulation Between Adiponectin and Plasminogen Activator-inhibitor-1 in 3T3-L1 Cells

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Abstract. *Background: Adiponectin (APN) and plasminogen activator inhibitor-1 (Pai-1) are adipocytokines, and low levels of serum APN and high levels of PAI-1 are observed in obese patients. Moreover, both APN and Pai-1 are known to be involved in colorectal carcinogenesis. Recently, we demonstrated that serum Pai-1 levels are elevated in APN-deficient mice. We hypothesized that Pai-1 expression levels could be depressed by APN. Thus, we aimed to clarify the bi-directional regulatory mechanisms between APN and Pai-1. Materials and Methods: We investigated the expression levels of APN and Pai-1 during 3T3-L1 pre-adipocyte differentiation, and examined the role of AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor (PPAR)-γ on APN and Pai-1 expression at early and late differentiation stages. Results: In the early phase of differentiation, Pai-1 expression increased and APN slightly decreased. Reduction of Pai-1 or activation of PPARγ resulted in elevation of APN, and supplementation of APN with activation of AMPK resulted in reduction of Pai-1. In the late phase of differentiation, APN increased its expression and Pai-1 decreased. Supplementation of Pai-1 resulted in a slight reduction of APN. Conclusion: It is suggested that APN and Pai-1 expressions are inversely-regulated. Understanding of the regulatory system between APN and Pai-1 may lead to finding novel methods for colorectal cancer prevention.*

Adiponectin (APN; 30 kDa protein) is one of the adipocytokines discovered in adipose tissue (1), and abundant amounts of APN are detected in plasma (3-30 μg/ml). A decrease of APN levels is associated with insulin-resistant type-2 diabetes, coronary artery disease, and the development of cancer, including colorectal cancer (2-6). There are two APN receptors, AdipoR1 and AdipoR2 (7). The physiological function of APN is evoked by binding to these receptors. It is known that AdipoR1 activates AMP-activated protein kinase (AMPK) and AdipoR2 activates peroxisome proliferator-activated receptor-α (PPARα) (8, 9).

We have been studying the involvement of APN in colorectal cancer risk. *Adenomatous Polyposis Coli* (*Apc*)-deficient Min mice ($Apc^{Min/+}$), a model of familial adenomatous polyposis (FAP), with APN deficiency were used to investigate the effects of *APN* knockout on intestinal polyp development. APN-deficient *Min* mice show a 2- or 3-fold increase in the total number of intestinal polyps developed compared with APN wild-type *Min* mice, regardless of gender (10). APN-deficient C57BL/6J mice treated with azoxymethane (AOM) demonstrated increased incidence and multiplicity of colorectal tumors, including adenomas and adenocarcinomas. *Min* mice exhibited an increase in serum plasminogen activator inhibitor-1 (Pai-1) levels with decreasing expression levels of *APN*. In addition, the tendency for elevation of serum Pai-1 levels was observed with APN-deficiency in C57BL/6J mice at the age of 55 weeks (10).

Pai-1 is one of the adipocytokines whose levels increase with obesity. Pai-1 directly inhibits tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). tPA and uPA activate plasminogen to produce plasmin through serine protease activity, and physiologically breakdown blood clots. Pai-1 is also reported to possess/exhibit multifunctional factors. Although the molecular mechanisms are not fully-established, Pai-1 was found to modulate cell proliferation and stimulate angiogenesis (11, 12). PAI-1 is known to be induced by triglyceride (TG), very low-density lipoprotein (TG-rich lipoprotein), transforming growth factor-β (TGFβ), various

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growth factors, tumor suppressor p53, nuclear factor kappa B (NFκB) and Wnt signaling (13-17), all of which are plausibly involved in carcinogenesis.

Adipocytokines can affect each other. Among them, APN is known to act as a major regulator of other adipocytokines. For instance, APN stimulates AMPK in the hypothalamus to promote food intake under starvation conditions and inhibit leptin activation (18). In peripheral tissues, especially in skeletal muscle, APN activates AMPK, insulin receptor substrate-1 and fatty acid transport protein-1, to stimulate fatty acid combustion and glucose intake. It is interesting that these types of activation can be inhibited by tumor necrosis factor α (TNFα), another adipocytokine. Thus, it is assumed that APN deficiency affects the action elicited by other adipocytokines or the production of other adipocytokines, such as Pai-1. Therefore, we hypothesized that Pai-1 expression levels might also be depressed by APN.

In the present study, we aimed to clarify the bi-directional regulatory mechanisms between APN and Pai-1. We investigated the expression levels of APN and Pai-1 during 3T3-L1 pre-adipocyte differentiation, and examined the role of AMPK and PPARγ on APN and Pai-1 expression at the early and late differentiation stage. We demonstrated that APN can suppress Pai-1 expression through activation of AMPK, and Pai-1 can suppress APN expression through inhibition of a transcription factor, PPARγ.

Materials and Methods

Cell culture and induction of adipocyte maturation in the 3T3-L1 cell line. 3T3-L1 cells (JCRB Cell Bank, Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, UT, USA) (basal medium). Induction of differentiation into adipocyte phenotypes was performed by treating confluent cells with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich Co., St. Louis, MO, USA), 1 μM dexamethasone (Sigma-Aldrich) and 1.6 μM insulin (Life Technologies, Co., Carlsbad, CA, USA) in basal medium for two days (Figure 1A). After the treatment, the medium was replaced by basal medium, and the cells were incubated for three days (indicated as day 5 in Figure 1A) and 16 days (indicated as day 18 in Figure 1A).

Mouse recombinant adiponectin (R&D Systems Inc., Minneapolis, MN, USA), metformin (Wako Pure Chemical Industnes, Osaka, Japan), troglitazone (Sigma-Aldrich) and PNU74654 (Wnt-I; Sigma-Aldrich) were applied to adipocyte cells on day 2 and the cells were incubated until day 5. Mouse recombinant Pai-1 (Merck, Darmstadt, Germany) was applied to adipocyte cells on day 15 and the cells were incubated until day 18.

Mouse fat tissue samples. Five abdominal fat tissue samples from 15-week-old male APN-deficient mice and APN wild-type mice were obtained from our previous experiment reported elsewhere (10).

Western blot analysis. Protein expression was analyzed by western blot. Cells (2×10^5) were seeded in 24-well plates. After treatment, cells were lysed in 100 μl lysis buffer [0.0625 M Tris-HCl (pH 6.8), 20% 2-mercaptoethanol, 10% glycerol, 5% sodium dodecyl sulfate] and Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific Inc., Waltham, MA, USA) added. Equal amounts of protein were separated in 5-20% gradient polyacrylamide gel electrophoresis-sodium dodecyl sulfate gels and transferred onto polyvinylidene difluoride membranes (Merck-Millipore, Billpore, MA, USA). Antibodies against p-AMPK and AMPK (Cell Signaling Technology, Danvers, MA, USA) were used at a 1:1,000 and 1:2,000 dilution, respectively. Blots were developed with enhanced chemiluminescence western blotting detection reagents (GE Healthcare, Buckingham Shire, UK).

Quantification of mRNA expression by quantitative real-time Polymerase Chain Reaction (qRT-PCR). Total RNA was isolated from cultured adipocyte and tissue samples using TRIzol Reagent (Invitrogen, Grand Island, NY, USA). One-microgram aliquots in a final volume of 20 μl were used for synthesis of cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was carried out using a CFX96™ (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with FastStart Universal SYBR Green MIX (×2) (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Primers for mouse *APN* (5' AGGATGCTA CTGTTGCAAGCTCTC, 5' CAGTCAGTTGG TATCATGGTAGAG), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; 5' TTGTCTC CTGCGACTTCA, 5' CACCACCCTGT TGCTGTA), Pai-1 (5' ACAGCCTTTGTCATCTCAGCC, 5' AGGG TTGCACTAAACAT GTCAG) were employed. The data were normalized by *GAPDH*. To assess the specificity of each primer set, amplicons generated from the PCR reaction were analyzed for melting curves.

Statistical analysis. Statistical analysis was performed using Student's *t*-test. Differences were considered to be statistically significant at $p<0.05$.

Results

Difference in APN and Pai-1 expression pattern during 3T3-L1 pre-adipocyte differentiation. 3T3-L1 is a suitable cell line to examine differences in molecular change during pre-adipocyte differentiation. Thus, expression levels of APN and Pai-1 were examined in 3T3-L1 cells at day 5 after the initiation of differentiation (early phase) and at day 18 after the initiation of differentiation (late phase). In the early phase, Pai-1 mRNA levels were significantly higher compared to those of undifferentiated 3T3-L1 cells (Figure 1B). In the late phase, *Pai-1* mRNA levels were significantly lower than those of undifferentiated 3T3-L1 cells. Comparing the expression levels of Pai-1 in early and late phases, an obvious reduction was observed in differentiated 3T3-L1 cells, while a slight induction of Pai-1 was observed in undifferentiated 3T3-L1 cells (Figure 1B). Comparing day 5 and day18, induction of APN was observed in both undifferentiated and differentiated 3T3-L1 cells in the late phase (Figure 1C). *APN* mRNA levels in differentiated cells tended to be lower compared to those of undifferentiated 3T3-L1 cells in the early phase, and higher when cells were in the late phase (Figure 1C and D).

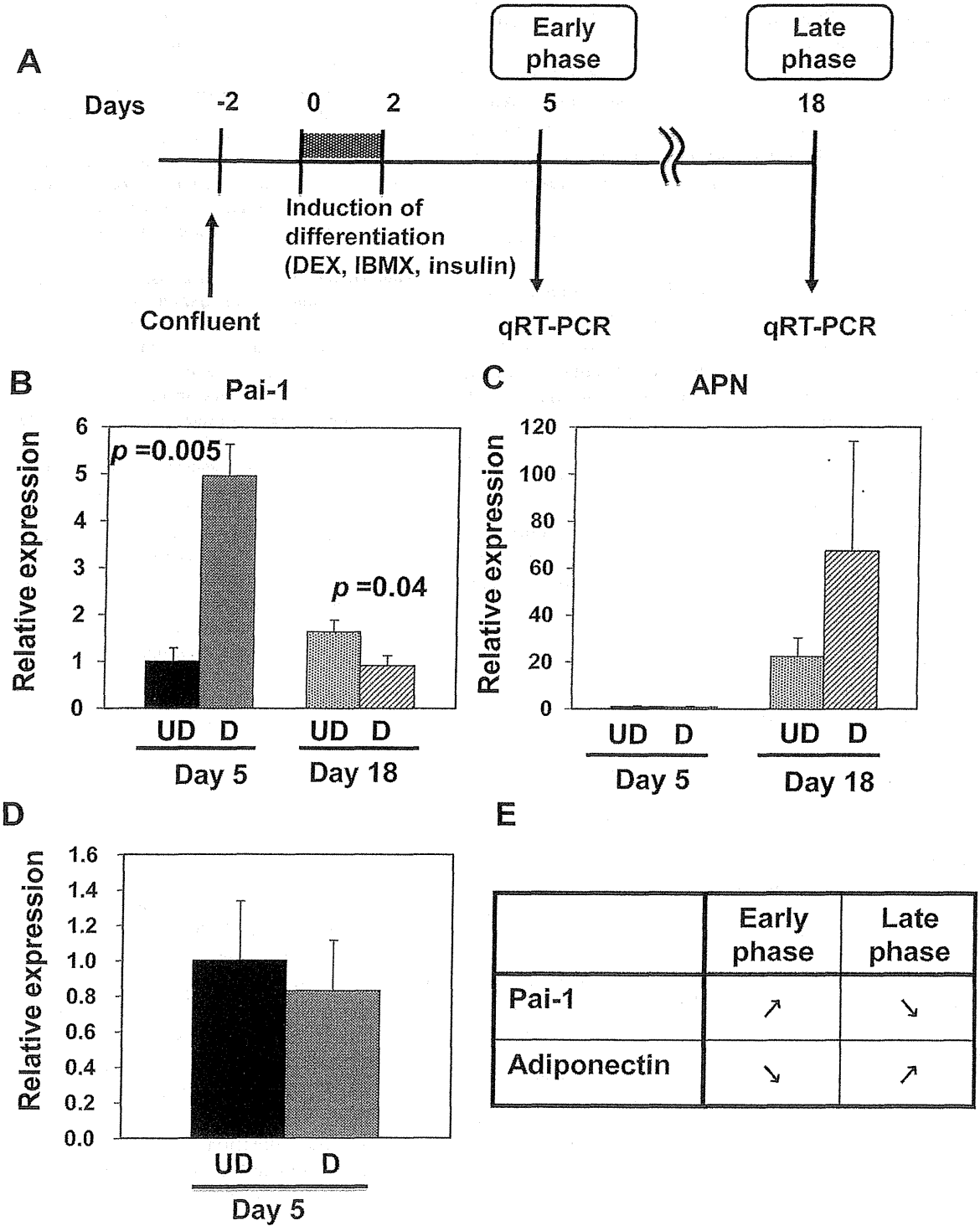

Figure 1. *Adiponecti n(APN) and plasminogen activator inhibitor-1(Pai-1) expression levels in 3T3-L1 pre-adipocytes. A: Illustration of differentiation protocol for 3T3-L1 pre-adipocytes is shown. Day 5 after the initiation of differentiation is defined as the 'early phase' and day 18 is defined as the 'late phase'. Quantitative real time-polymerase chain reaction (qRT-PCR) for Pai-1 (B) and APN (C) was performed at day 5 and day 18. The data are normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative Pai-1 and APN mRNA expression levels are plotted as the ratio of the untreated and undifferentiated control culture values. Data are means±SD (n=3). Similar results were obtained from more than three separate experiments. D: The data focused on low relative expression levels of Figure 1C. E: Summary of APN and Pai-1 expression patterns during 3T3-L1 pre-adipocyte differentiation. DEX, Dexamethasone; D, differentiated; IBMX, 3-isobutyl-1-methylxanthine; UD, undifferentiated.*

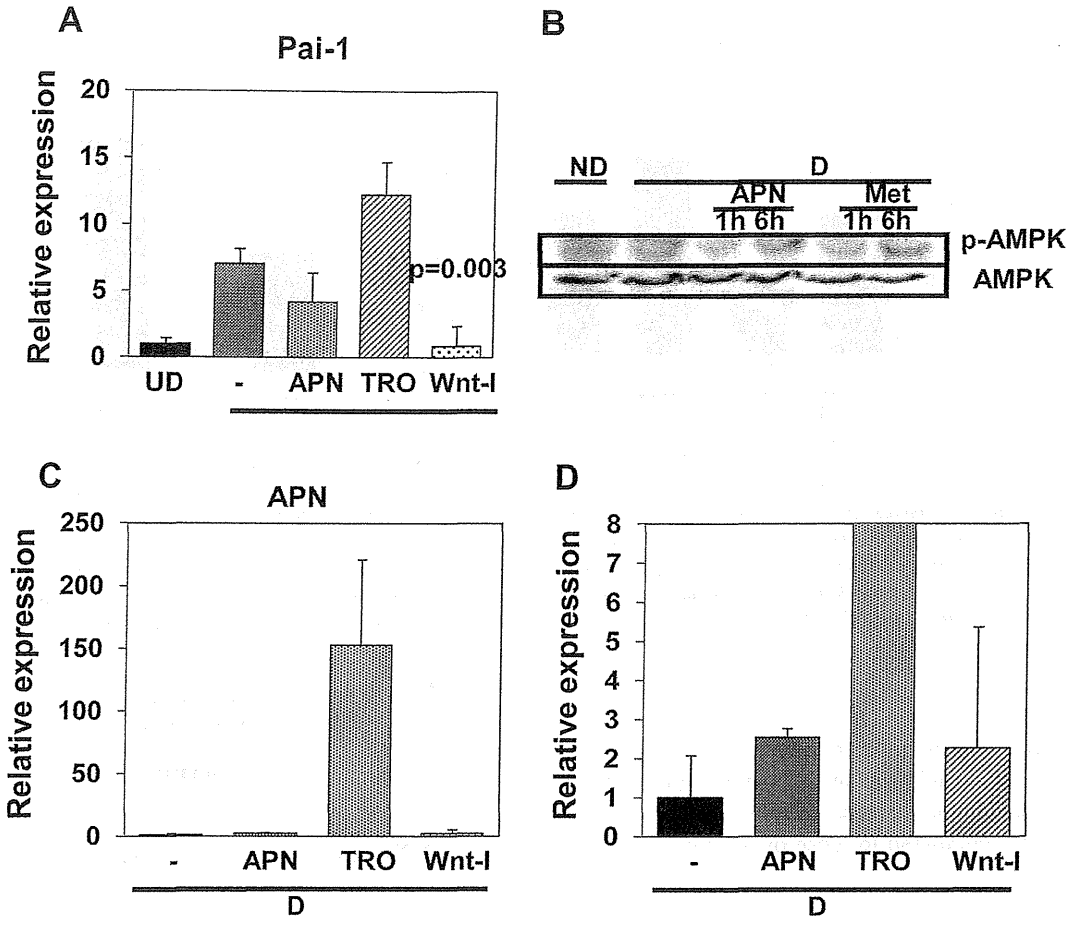

Figure 2. *Effects of adiponectin (APN), peroxisome proliferator-activated receptor (PPAR)γ ligand and Wnt inhibitor on 3T3-L1 cells at the early stage of differentiation. At day 2 after the initiation of differentiation, 3T3-L1 cells were treated with mouse recombinant protein APN (10 μg/ml), troglitazone (TRO), PPARγ ligand (10 μM) and Wnt inhibitor PNU74654 (20 μM) for three days. Quantitative real time-polymerase chain reaction (qRT-PCR) for Pai-1 (A) and APN (C and D) was performed. Relative Pai-1 and APN mRNA expression levels are plotted as the ratio of the untreated and undifferentiated control culture value. Data are means±SD (n=3). Similar results were obtained from three separate experiments. B: 3T3-L1 cells were treated with APN (10 μg/ml) and metformin (MET) an AMPK activator (5 mM) for 1 or 6 h, and AMPK and phosphorylated AMPK were examined by western blot. D: The data focused on low relative expression levels of Figure 1C. D, differentiated; UD, undifferentiated.*

Correlation between adiponectin and Pai-1 in the early phase. To clarify the relation between APN and Pai-1, 3T3-L1 cells were treated with APN at a dose of 10 μg/ml on day 2, after the initiation of differentiation. At the early-phase time point (day 5), high *Pai-1* mRNA expression levels were observed and it was found that APN could slightly reduce Pai-1 expression levels compared to those differentiated cells not treated with APN (Figure 2A). Phosphorylation of AMPK was confirmed by western blotting after six hours treatment with 10 μg/ml APN and 5 mM metformin, used as a positive control, at day 2 after the initiation of differentiation (Figure 2B). In addition, we tried to induce an increase in APN expression by treatment with troglitazone, a PPARγ ligand. As expected, treatment with 10 μM troglitazone markedly induced APN as shown in Figure 2C. However, treatment with troglitazone did not suppress but rather increased Pai-1 expression. On the other hand, we tried to reduce the high levels of Pai-1 by inhibiting Wnt/β-catenin signaling. PNU74654, a Wnt inhibitor, at a dose of 20 μM successfully suppressed Pai-1 expression levels (Figure 2A). APN expression levels under this treatment were examined, and almost a 2-fold elevation was observed (Figure 2C and D).

Correlation between adiponectin and Pai-1 in the late phase. To clarify the relation between APN and Pai-1 in the late phase, Pai-1 at a dose of 1 μg/ml was added to the medium at 15 days after the initiation of differentiation. At the late-phase time point (day 18), APN expression was slightly reduced by Pai-1 treatment compared to those of untreated

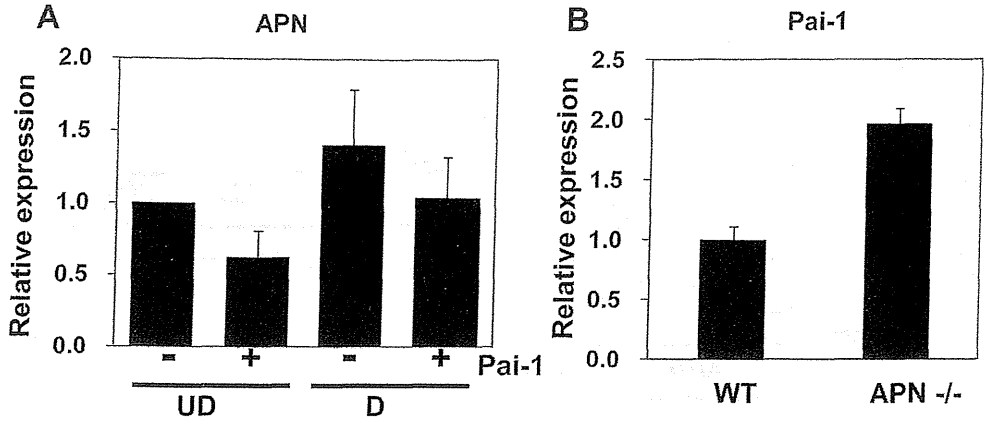

Figure 3. *Effects of plasminogen activator inhibitor-1 (Pai-1) on adiponectin (APN) mRNA levels and expression of Pai-1 in adiponectin-deficient mice. A: 3T3-L1 pre-adipocytes were treated with 1 μg/ml mouse recombinant Pai-1 at day 15 after the initiation of differentiation, and cells were collected for Quantitative real time-polymerase chain reaction (qRT-PCR) analysis at day 18. Relative APN mRNA expression levels are plotted as the ratio of the unstimulated-control culture value. Data are means±SD (n=3). Similar results were obtained from more than two separate experiments. B: qRT-PCR was performed on abdominal fat tissue from 12-week-old male adiponectin homozygous knockout mice (C57BL/6J background; n=5) and its control wild-type (WT) mice (n=5) as described in the Materials and Methods. Relative Pai-1 mRNA expression levels are plotted as the ratio of the value for the wild-type control fat tissue. Data are means±SD. APN(-/-), Homozygous adiponectin knockout mice; D, differentiated; UD, undifferentiated.*

differentiated and undifferentiated cells (Figure 3A). Furthermore, we confirmed an almost doubling of Pai-1 levels in the abdominal adipose tissue in *APN* homozygous knock-out mice compared to those of *APN* wild-type mice (Figure 3B).

Discussion

The present study demonstrated seesaw patterns of APN and Pai-1 expression in the different stages of pre-adipocyte differentiation. Moreover, bi-directional regulation observed between APN and Pai-1 may be through activation of AMPK and PPARγ (Figure 4).

In the early phase of 3T3-L1 cell differentiation, Pai-1 expression increased and APN slightly decreased. Besides, the late phase of differentiation showed low Pai-1 and high APN (Figure 1E). PPARγ, sterol regulatory element-binding protein-1c (SREBP-1c) and CCAAT/enhancer-binding proteins (C/EBP) are known to be involved in the early changes during pre-adipocyte differentiation (19). In the late phase of pre-adipocyte differentiation, the canonical Wnt signaling pathway reduces adipogenesis (19). These signalings might affect expression patterns observed for APN and Pai-1.

Indeed, a PPARγ ligand remarkably induced APN expression in this study, especially in the early phase (Figure 4A). Another PPARγ ligand, pioglitazone was also found to induce APN expression (20). Of note, Pai-1 is reported to suppress PPARγ expression (21). In this study, APN induction did not effectively lower Pai-1, but addition of 10 μg/ml APN to the culture medium did reduce Pai-1 expression. Concentrations of APN

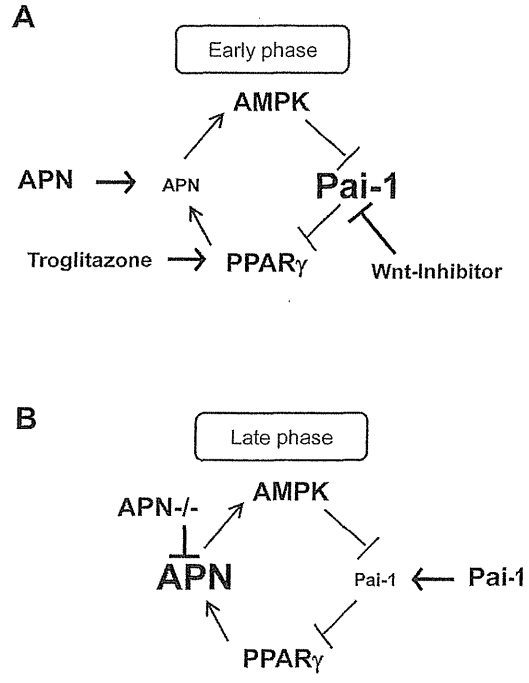

Figure 4. *Proposed mechanism for the seesaw regulation between adiponectin (APN) and plasminogen activator inhibitor-1 (Pai-1). A: Low APN and high Pai-1 expression levels in the early differentiation phase of 3T3-L1 cells may be regulated by AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor (PPAR)γ. Wnt inhibitor was used for suppression of Pai-1. Troglitazone was used for activation of PPARγ. APN was used for activation of AMPK. B: High APN and low Pai-1 expression levels in the late differentiation phase of 3T3-L1 cells may also be regulated by AMPK and PPARγ. Pai-1 was used for suppression of PPARγ. Abdominal fat tissue from APN-deficient mice (APN-/-) was used to determine the effects of APN deficiency on Pai-1 expression levels (→induction; —| suppression/inhibition).*

detected in plasma range from 3-30 μg/ml. Thus, a biologically appropriate dose might be used in this study. It has been reported that the activation of AMPK leads to the inhibition of adipogenesis (22). AMPK activation by APN resulted in suppression of Pai-1 expression. Similar findings were obtained in our recent experiment (10), in which APN-deficiency evoked hepatic Pai-1 induction. These findings suggest that in addition to the Pai-1-suppressive function of TNFα, APN-induced AMPK activation acts as a more direct physiological suppressor of Pai-1. Moreover, Wnt signal inhibitors lowered Pai-1 expression in the early phase. Pai-1 is reported to be a downstream target of Wnt/β-catenin signaling (17), and this might be the reason why APN is induced by a Wnt inhibitor. Summarizing effects in the early phase in 3T3-L1 cells, reduction of Pai-1 resulted in elevation of APN, and supplementation of APN resulted in reduction of Pai-1 (Figure 4A).

In the later phase of pre-adipocyte differentiation, low expression of Pai-1 and high expression of APN were observed. Addition of 1 μg/ml Pai-1 in the culture medium slightly reduced APN expression. Generally, the concentration of PAI-1 detected in human plasma is lower than 50 ng/ml. This dose may not be a biologically-appropriate dose, but may partly explain Pai-1 functions on specific occasions, such as in a localized area in the late phase of differentiation. We also examined the effect of APN-knockout conditions on Pai-1 expression using abdominal fat tissue samples from APN homozygous knock-out mice. Pai-1 expression was observed at a high level compared to that of fat tissue from APN wild-type mice. In the late phase of 3T3-L1 cells, supplementation of Pai-1 resulted in a slight reduction of APN (Figure 4B).

Both APN and Pai-1 are known to be involved in colorectal carcinogenesis. An absence of APN results in an increase of intestinal polyp development in *Min* mice (10), while a PAI-1 inhibitor was reported to reduce intestinal polyp development in *Min* mice (23). It is assumed that both induction of APN and inhibition/suppression of Pai-1 may be a useful approach in colorectal cancer prevention. Here, we have demonstrated a seesaw pattern of regulation between APN and Pai-1. Further studies are required to identify more direct regulatory mechanisms between APN and Pai-1, and the molecular targets identified might be utilized as novel chemopreventive targets.

Conflicts of Interest

None.

Acknowledgements

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ORIGINAL ARTICLE

The preventive effects of low-dose enteric-coated aspirin tablets on the development of colorectal tumours in Asian patients: a randomised trial

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ABSTRACT

Objective To evaluate the influence of low-dose, enteric-coated aspirin tablets (100 mg/day for 2 years) on colorectal tumour recurrence in Asian patients with single/multiple colorectal tumours excised by endoscopy.

Design A double-blinded, randomised, placebo-controlled multicentre clinical trial was conducted.

Participants 311 subjects with single/multiple colorectal adenomas and adenocarcinomas excised by endoscopy were enrolled in the study (152 patients in the aspirin group and 159 patients in the placebo group). Enrolment began at the hospitals (n=19) in 2007 and was completed in 2009.

Results The subjects treated with aspirin displayed reduced colorectal tumourigenesis and primary endpoints with an adjusted OR of 0.60 (95% CI 0.36 to 0.98) compared with the subjects in the placebo group. Subgroup analysis revealed that subjects who were non-smokers, defined as those who had smoked in the past or who had never smoked, had a marked reduction in the number of recurrent tumours in the aspirin-treated group. The adjusted OR for aspirin treatment in non-smokers was 0.37 (CI 0.21 to 0.68, p<0.05). Interestingly, the use of aspirin in smokers resulted in an increased risk, with an OR of 3.44. In addition, no severe adverse effects were observed in either group.

Conclusions Low-dose, enteric-coated aspirin tablets reduced colorectal tumour recurrence in an Asian population. The results are consistent with those obtained from other randomised controlled trials in Western countries.

The clinical trial registry website and the clinical trial number http://www.umin.ac.jp (number UMIN000000697).

Significance of this study

What is already known on this subject?

- A considerable amount of evidence regarding the utility of aspirin as a cancer chemopreventive agent has been generated in Western populations.
- The evidence regarding aspirin as a cancer chemopreventive agent in Asian populations is limited. Moreover, no cancer chemopreventive drugs have been approved in Japan.
- The advantages of aspirin as a cancer chemopreventive agent are well recognised, as it has been in clinical use for a long period of time. In addition, aspirin's adverse effects and cost-effectiveness are well known.

What are the new findings?

- We are the first to report the efficacy of low-dose, enteric-coated aspirin tablets in the suppression of colorectal tumour recurrence in Asian patients; these findings are consistent with the observations of other aspirin adenoma trials in Western populations.
- We report the safety of low-dose, enteric-coated aspirin tablets administered to patients as a cancer chemopreventive agent for 2 years.

How might it impact on clinical practice in the foreseeable future?

- The evidence that aspirin is effective in the reduction of colorectal tumour recurrence in Asian patients may impact cancer preventive strategies in Japan and other Asian countries, including Korea and China.

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INTRODUCTION

Among chemopreventive interventions, aspirin (acetylsalicylic acid) has been examined in numerous trials that support its suppressive effect on colorectal cancer (CRC) development. Aspirin is a synthetic medicine based on the structure of salicylates, which are commonly found in fruits and vegetables. Aspirin's antineoplastic effects have been mechanistically explained by its cyclooxygenase (COX) inhibitory activity. The use of aspirin as a cancer chemopreventive agent is advantageous because it has a long history of clinical use

and its adverse effects are well known. Moreover, the cost-effectiveness of aspirin administration to prevent other diseases, such as cardiovascular disease, has also been demonstrated.[1]

An early prospective cohort study of 662 424 adults (the Cancer Prevention Study II cohort) demonstrated that the CRC death rate decreased with frequent aspirin use. The decreased relative risk (RR) of CRC among frequent aspirin users (≥16 times/month for at least 1 year with doses greater than 160 mg) was 0.60 (95% CI 0.4 to 0.89) in men and 0.58 (95% CI 0.37 to 0.9) in women.[2] An updated analysis of this cohort (the Cancer Prevention Study II Nutrition cohort) demonstrated that long-term daily aspirin use (≥325 mg/day for ≥5 years) is associated with reduced incidence of CRC compared with non-users (RR=0.68, 95% CI 0.52 to 0.90 among men and women collectively).[3 4] The factors that may affect the impact of aspirin include the population, dose of aspirin and duration of intervention.[4] In the general population, trials of 75–325 mg/day aspirin for 3 years reduced the risk of recurrent of colorectal adenoma by 17%.[5] Moreover, the use of aspirin for 5 years or longer reduced the incidence and mortality of CRC by 30%–40% after 20-year follow-up.[6]

A considerable amount of evidence on the utility of aspirin has been generated in Western populations; however, the evidence for aspirin as a cancer chemopreventive agent in Asian populations is limited. Thus, it is important to present evidence that aspirin is also effective as a cancer chemopreventive agent in Asian populations.

We recently reported a double-blinded, randomised, placebo-controlled clinical trial of a high-risk CRC group, familial adenomatous polyposis, to evaluate the effect of low-dose, enteric-coated aspirin tablets. Secondary endpoint data from the trial revealed that subjects with a mean baseline polyp diameter of <2 mm administered aspirin displayed a significant reduction in mean polyp size.[7]

We investigated the effects of low-dose, enteric-coated aspirin tablets administered for 2 years in a double-blinded, randomised, placebo-controlled clinical trial in patients with a single/multiple colorectal adenomas and/or adenocarcinomas with invasions confined to the mucosa and excision by endoscopy. This population was considered to be a high-risk colorectal tumour group. Low-dose, enteric-coated aspirin tablets (100 mg/day) were chosen for the study because low-dose aspirin may circumvent the risk of upper GI toxicity.[8] In addition, the enteric coating may decrease gastric mucosal damage, as demonstrated in the MAJIC study targeting high-risk cardiovascular Japanese patients[9] as well as other short-term endoscopic studies.[10]

Here, we report the efficacy and safety of low-dose, enteric-coated aspirin tablets in the suppression of colorectal tumour recurrence in Asian patients with colorectal adenomas and/or adenocarcinomas with confined mucosal invasions that were excised by endoscopy.

METHODS

Trial methodology

In this double-blinded (both subjects and investigators), randomised, placebo-controlled trial using low-dose, enteric-coated aspirin tablets, the subjects received either 100 mg/day aspirin or placebo for 2 years. Each case was randomised by investigators using a computer-aided system from the Medical Research Support website. Using a minimisation algorithm, the primary examination selection was balanced with respect to three stratification variables: institution, age (≤60 and >60 years) and sex (male or female). The website was only available to the trial investigators. Subject enrollment and intervention assignment began at each hospital in January 2007, and the trial ended in July 2009. To further evaluate the effects of aspirin, follow-up for more than 2 years after the randomised trial was also planned. An Ethics Monitoring Committee was established for this multicentre trial (n=19) that was primarily based at Osaka Central Hospital. A system to ensure continuous follow-up of adverse events was also established. All hospitals participating in this trial obtained approval from their own ethics committees. This trial is registered and details are available at http://www.umin.ac.jp (number UMIN000000697), where the full trial protocol can be accessed.

Trial population

The trial population (n=389) consisted of patients with single/multiple colorectal adenomas and/or adenocarcinomas with invasions confined to the mucosa. The colorectal tumours of all subjects participating in the trial were excised by endoscopy before the trial start. An endoscopic examination was performed twice before the start of the trial; the examinations occurred at an average of 488.4 ±472 (mean±SD) days apart to confirm that all colorectal tumours were excised. All of the subjects were Asian men or women 40–70-years-old living in Japan. The following are exclusion criteria for the trial: (1) patients with familial adenomatous polyposis, Lynch syndrome or colorectal resection; (2) patients currently taking an antithrombotic or anticoagulant, including aspirin; (3) individuals with a history of stroke or gastric/duodenal ulcers (with the exception of patients with confirmed scars resulting from the successful eradication of *Helicobacter pylori*); (4) patients with IBD, haemorrhagic diverticulitis or haemorrhagic tendency; (5) patients with a platelet count of ≤100 000/mm^3 or abnormal prothrombin time; (6) patients with a known aspirin allergy; (7) patients currently taking an anticancer drug; (8) pregnant patients or those who planned to become pregnant during the trial period; and (9) patients taking non-steroidal anti-inflammatory drugs (NSAIDs) for pain relief more than thrice weekly. We calculated that 266 randomised patients would achieve an 80% power (with a 5% type I error) to detect a 40% difference in the recurrence rate of adenoma given a 40% risk of recurrence in the placebo group.[11] However, data were unavailable to calculate an appropriate number of individuals to recruit from the Asian population; therefore, we set our recruitment goal in the initial aspirin protocol to 700 randomised patients.

Consent interviews were performed individually, and written informed consent was obtained from all patients.

Investigational drug

Low-dose, enteric-coated aspirin tablets (100 mg per tablet) and the placebo tablets were kindly provided by Bayer Pharma AG (Leverkusen, Germany) and imported into Japan. The trial was financed by research funding from the Ministry of Health, Labor and Welfare, not by Bayer Pharma AG. We signed an agreement to certify that no conflicts of interest with Bayer Pharma AG existed. The investigational drugs were placed in blister packages (calendar sheets of 31 tablets), and both sides of the package were aluminium-laminated.

Trial questionnaire

At the time of trial enrolment, the height, body weight, medical history, smoking history, alcohol ingestion and use of NSAIDs were investigated for each patient using a questionnaire. In addition, data regarding everyday meals were collected using a self-administered food-frequency questionnaire developed by the Department of Health Promotion and Preventive Medicine,

Nagoya-City University Graduate School of Medical Science, Aichi, Japan.[12] Non-smokers were defined as people who had smoked in the past or never smoked. Occasional drinkers were defined as people who drank less than twice a week.

To ensure the accurate characterisation of adverse effects and evaluation of tolerability, the subjects were asked to keep a treatment diary that documented their conditions during treatment, such as drug compliance and medical conditions, and a blister sheet was sent to the data centre every month.

Trial endpoints

Colonoscopy was performed at least three times, twice before the start of the trial and once at the end of the trial. All the patients were given an oral lavage solution for colonic cleansing at the time of the colonoscopy for clear imaging, and a medical colonoscopy specialist carefully examined the patients from the rectum to the cecum. The final endoscopy examination was performed 2 years after the start of the trial. Recurrent tumours were further diagnosed by histology after tumour excision. The primary endpoint was the incidence of adenoma or adenocarcinoma recurrence. The data were analysed using logistic regression and ORs, and general factors, such as sex, age and the tumour number before the trial, were used to adjust occasional deviation during the randomised allocation. Each tumour was removed and examined histologically by a pathologist. Tumours were classified as adenomas or adenocarcinomas according to the 'Japanese Classification of Colorectal Carcinoma' criteria. The secondary endpoints included recurring tumour number, size and histology as well as the effects of lifestyle, such as smoking and alcohol drinking, and the frequency of adverse effects.

Statistical analysis

The baseline characteristics of the two arms were compared using the χ^2 test or the t test. The adverse effect rates of both arms were compared using the χ^2 test. If needed, Fisher's exact probability was applied due to sparse data in a table. To adjust for potential confounding effects at baseline, logistic regression was performed.

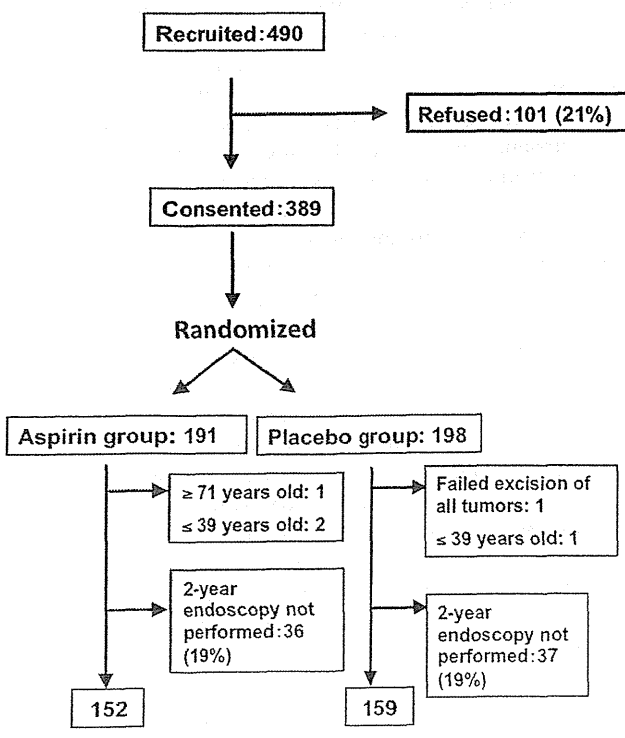

Figure 1 Flowchart of subject recruitment.

We also examined the effect modification (interaction) of several factors, such as (1) sex, (2) age, (3) smoking and (4) alcohol drinking, on the main effect of aspirin by adding an interaction term to the logistic regression. In this analysis, we determined the ORs of the subgroups of the above factors and the difference in the ORs of the subgroups.

All statistical analyses were based on the intention-to-treat and performed using PC-SAS (V.9.3; SAS Inc., Cary, North Carolina, USA), with $p<0.05$ considered statistically significant.

RESULTS

Characteristics of the trial subjects

A total of 490 patients were screened, and 389 patients provided informed consent. Subject enrolment began in January 2007, and the trial ended in July 2009. Subject recruitment ended according to the planned time schedule. After randomisation, the aspirin and placebo group consisted of 191 and 198 subjects, respectively. At the end of the trial, 152 subjects from the aspirin group and 159 subjects from the placebo group underwent a 2-year follow-up endoscopy examination (figure 1). The characteristics of the subjects in the aspirin and placebo groups after randomisation are displayed in table 1. No significant differences between the two groups were observed with regard to the following characteristics: age, sex, smoking status, alcohol drinking status, height, weight, body mass index, tumour number upon entry into the trial, past history of CRC with invasion confined to the mucosa, treatment period, compliance (ie, whether patients correctly take medicine and follow the doctors' instructions (data not shown)), surgical history (data not shown) and family history of CRC (data not shown). The serum concentrations of alanine transaminase, aspartate amino transferase, γ-glutamyl transpeptidase and triglycerides were almost identical between the groups (data not shown).

Colorectal tumour recurrence as the primary endpoint

In total, 96 patients did not experience colorectal tumour recurrence in the aspirin group (total 152), and 86 patients in the placebo group (total 159) did not recur. In crude analyses, the subjects in the aspirin group tended to demonstrate a reduced number of colorectal tumours, which was the primary endpoint, compared with subjects in the placebo group. The OR was 0.69 (95% CI

Table 1 Characteristics of the two groups

	Aspirin		Placebo	
Number	152		159	
Age	60.0	±7.3*	60.5	±6.6
Sex				
Male	121	(79.6%)	125	(78.6%)
Smoking				
Current smokers	45	(29.6%)	34	(21.4%)
Alcohol				
Drinker†	83	(54.6%)	92	(57.9%)
Height	164.7	±6.8	165.5	±7.3
Weight	64.3	±9.7	65.6	±10.1
BMI‡	23.6	±2.7	23.9	±2.8
Number of tumours upon trial entry	5.3	±5.7	5.1	±7.0
Past CRC history	40	(26.3%)	39	(24.5%)
Treatment period	751	±67 days	764	±90 days

*SD.
†Alcohol drinker: drinks more than three times a week.
‡Body mass index (BMI)=Weight (kg)/height (m) squared.
CRC, colorectal cancer.

Table 2 The effects of aspirin on colorectal tumour development in smokers

Subanalysis	No. of subjects with (+) or without (−) colorectal tumour			Adjusted OR (95% CI)
	−	+	Total	
Current smoker				
Placebo group	26	19	45	1
Aspirin group	14	20	34	3.45 (1.12 to 10.64), p=0.03
Non-smoker*				
Placebo group	60	54	114	1
Aspirin group	82	36	118	0.37 (0.21 to 0.68), p=0.01

Adjusted OR, OR is adjusted by sex, age and the number of tumours prior to the trial.
*Non-smoker: never smoked and former smokers.

0.44 to 1.08); despite a marginal difference, the value was not statistically significant. To adjust for potential confounders, such as sex, age and the number of recurrent tumours, we performed logistic regression and obtained a significant OR value of 0.60 (95% CI 0.36 to 0.98). The OR for the number of recurrent tumours <4 in the aspirin group was 0.34 (0.09 to 1.26), and the OR for a tumour >3 mm in longitudinal diameter was 0.86 (0.63 to 1.16), but the value was not statistically significant.

The effects of smoking and drinking on colorectal tumour recurrence

Using a logistic regression with smoking as the interaction term and aspirin as the effect, we observed that smoking displays strong effect modification on the main effect of aspirin (p for interaction=0.004). Namely, the OR for non-smokers was 0.37 (95% CI 0.21 to 0.68), and this value was significantly different from the OR for smokers (OR 3.44, 95% CI 1.12 to 10.64) after adjustment for age, sex and the number of tumours (table 2). In contrast, no significant effect modification for sex (p=0.68), age (p=0.53) or alcohol consumption (p=0.32) was observed. With regard to sex, the OR was 0.48 (95% CI 0.15 to 1.55) and 0.63 (95% CI 0.36 to 1.08) among men and women, respectively. For age, the OR was 0.68 (95% CI 0.36 to 1.28) and 0.49 (95% CI 0.22 to 1.08) for subjects aged <60 and ≥60 years, respectively. For alcohol consumption, the OR was 0.72 (95% CI 0.37 to 1.40) and 0.44 (95% CI 0.21 to 0.95; p<0.05) for drinkers and occasional drinkers, respectively.

In addition, no severe adverse effects, such as cardiovascular events, were reported in either group. GI bleeding was not observed. Of note, colorectal adenocarcinomas were observed in four subjects: two cases from the aspirin group (one adenocarcinoma with invasion confined to the mucosa, and one adenocarcinoma with muscularis propria invasion) and two in the placebo group (two adenocarcinomas with invasion confined to the mucosa). The remaining tumours were tubular adenomas; villous adenomas were not identified. In addition, three high-grade dysplasias were detected; one case was observed in the aspirin group, and two cases were noted in the placebo group. The adenocarcinomas were 10–20 mm in diameter. The lesions were localised to the transverse colon (n=2), the descending colon (n=1) and the sigmoid colon (n=1).

DISCUSSION

In the present trial, we enrolled subjects with single/multiple colorectal adenomas and/or adenocarcinomas with invasions confined to the mucosa that were excised by endoscopy. Patients treated with low-dose, enteric-coated aspirin tablets for 2 years were shown to have a low risk of incidental colorectal tumour development, and this appeared to be reduced after adjustment for sex, age and the number of baseline tumours. Moreover, smoking significantly modified the preventive effect of aspirin.

In a meta-analysis of subjects with a history of colorectal adenoma or cancer in four randomised adenoma prevention trials (nearly 3000 patients), aspirin reduced the occurrence of advanced lesions (ie, tubulovillous adenomas, villous adenomas, adenomas ≥1 cm in diameter, adenomas with high-grade dysplasia or invasive cancer) by 28% (adenoma 17%; RR=0.83; 95% CI 0.72 to 0.96).[3] Our trial also demonstrated reduced adenoma occurrence (OR=0.69), and similar effects were obtained compared with the meta-analysis by Cole *et al*.[5] However, the ORs we used have a predictable effect on the comparison of the two sets of analyses. Regarding the limitations of our trial, the number of subjects enrolled is rather small, but the tumour recurrence results are consistent with previous studies. Thus, our data demonstrate that aspirin is also useful as a CRC chemopreventive agent in an Asian population. Of note, the first Asian adjuvant study (ASCOLT, NCT00565708) is ongoing, wherein patients with Dukes C or high risk Dukes B CRC are treated with aspirin (200 mg/day for 3 years).

Although the daily aspirin doses administered for vascular disease prevention are as effective as high-dose (1200 mg/day) aspirin,[11] analyses comparing moderate (300–325 mg/day) and lower (81–160 mg/day) aspirin doses trials (AFPPS[11] and APACC[13]) revealed that the reduced risk of all adenoma recurrence was only observed with lower doses.[5] Our trial using low-dose, enteric-coated aspirin tablets (100 mg/day) was designed in light of these trials, thereby confirming that low-dose, enteric-coated aspirin tablets effectively reduce recurrence. In addition, low-dose regimens may have an advantage in that the lower doses potentially reduce adverse effects. Aspirin has been reported to induce GI bleeding at a rate of 1–2 GI bleeds per 1000 person-years.[14] In our trial, no severe adverse effects due to aspirin treatment were observed.

Aspirin's antineoplastic effects are explained by COX-dependent and -independent mechanisms. In humans, aspirin inhibits COX-1 and COX-2 at high doses[15] and appears to effectively inhibit prostaglandin synthesis in the colon.[16] COX-independent mechanisms underlying aspirin's antineoplastic effects are attributed to the modulation of nuclear factor κ B: the induction of spermidine/spermine N1-acetyltransferase, caspase-8 and -9; and the activation of 5′ adenosine monophosphate-activated protein kinase, Erk and β-catenin.[17–23]

Despite copious information regarding aspirin's functions, the mechanism by which smoking negates aspirin's CRC chemopreventive effects remains unclear. A strong association between antiplatelet therapy resistance (aspirin resistance) and smoking has been reported. Specifically, a statistically significant interaction exists based on the multivariate analysis (risk ratio 11.47, CI 6.69 to 18.63, p<0.0001),[24] which is likely due to smoking-induced platelet hyperactivity and chronic inflammation.[25] In addition, smoking-induced decreased basal GI blood flow may also be involved.[26] Thus, it is suggested that smoking negated aspirin's chemopreventive effects in CRC. However, the evidence is limited. It is important to review and generate additional aspirin trial data to examine the association between NSAIDs use and smoking history and to determine whether the benefits of aspirin are limited to non-smokers.

In conclusion, although the size of this trial is small, the results are consistent with the observations of other aspirin adenoma trials; thus, aspirin may be useful for chemoprevention in Asian

patients with single/multiple colorectal tumours and no antecedent risk of GI bleeding. Several years of follow-up after a randomised trial are necessary to evaluate the effects of aspirin as proposed by the CAPP2 randomised trial.[27] Moreover, it would be informative to test aspirin in combination with other chemopreventive agents that have demonstrated effectiveness and agents that prevent GI bleeding (eg, proton-pump inhibitors).[28]

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Competing interests None.

Patient consent Obtained.

Ethics approval All hospitals participating in this trial obtained approval from their own ethics committees to conduct the trial.

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Tumor Suppressor APC Protein Is Essential in Mucosal Repair from Colonic Inflammation through Angiogenesis

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Mucosal repair after acute colonic inflammation is central to maintaining mucosal homeostasis. Failure of mucosal repair often leads to chronic inflammation, sometimes associated with inflammatory bowel disease (IBD). The adenomatous polyposis coli (*APC*) tumor suppressor gene regulates the Wnt signaling pathway, which is essential for epithelial development, and inactivation of *APC* facilitates colorectal cancer. Our previous study suggested that *APC* is involved in pathogenesis of colonic inflammation; however, its role in mucosal repair remains unknown. In this article, we report that colitis induced by dextran sodium sulfate persisted with delayed mucosal repair in Kyoto Apc Delta (KAD) rats lacking the APC C terminus. Defects in the repair process were accompanied by an absence of a fibrin layer covering damaged mucosa and reduced microvessel angiogenesis. APC was up-regulated in vascular endothelial cells (VECs) in inflamed mucosa in KAD and F344 (control) rats. The VECs of KAD rats revealed elevated cell adhesion and low-branched and short-length tube formation. We also found that DLG5, which is associated with IBD pathogenesis, was up-regulated in VECs in inflamed mucosa and interacted with the C terminus of APC. This finding suggests that loss of interaction between the APC C terminus and DLG5 affects VEC morphology and function and leads to persistence of colitis. Therefore, APC is essential for maintenance of intestinal mucosal homeostasis and can consequently contribute to IBD pathogenesis. *(Am J Pathol 2013, 182: 1263–1274; http://dx.doi.org/10.1016/j.ajpath.2012.12.005)*

Mucosal epithelial defense is an important system to prevent injuries induced by undigested substances, acid, ischemia, and microbial infection.[1,2] Once the mucosa is injured, the repair process—which is complex but primarily consists of the immune response, granulation tissue formation, angiogenesis, and epithelial regeneration—plays a central role to prevent further injuries.[3] Defects in such repair systems are a potential risk for persistent inflammation of the intestine or colon, which can lead to chronic inflammation, such as that seen in inflammatory bowel disease (IBD).

IBD, including ulcerative colitis and Crohn's disease, represents a chronic, relapsing and remitting inflammatory condition that affects individuals throughout life.[4] Patients with IBD are at risk of developing colorectal cancer.[5] Although it is widely accepted that genetic, environmental, and immunologic factors are involved,[6,7] the pathogenesis of IBD remains unclear. No completely effective therapeutic strategy has yet been established.

To investigate the pathogenesis of IBD, animal models of experimental colitis have been developed.[8] The dextran sodium sulfate (DSS) model is excellent for its resemblance to the clinical symptom of the IBD and for its ease of reproducibility and accessibility.[9] Providing drinking water containing DSS for several days induces colitis in rodents, which has characteristics similar to human ulcerative colitis, such as signs of diarrhea, gross rectal bleeding, weight loss, shortening of the colorectum, histologic features of multiple erosions, and inflammatory mucosal changes, occasionally including crypt abscess. Colitis is also predominant in the

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The KAD (F344-*Apc*^{m1Kyo}) rat has been deposited in the National Bioresource Project-Rat in Japan (Institute of Laboratory Animals, Kyoto University).

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tissues (3 μm thick) were made: two sections stained with H&E to permit histologic examination and with phosphotungstic acid hematoxylin (PTAH) for detection of fibrin. Other sections were used for IHC with an LSAB2 Kit (Dako, Glostrup, Denmark). Anti-APC monoclonal antibody (EP701Y; Abcam, Cambridge, UK) and anti-CD31/platelet–endothelial cell adhesion molecule 1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were used as primary antibodies.

Cell proliferation in the inflamed mucosa was accessed by determination of the labeling index for BrdU-positive cells. The number of BrdU-positive cells was counted in at least 20 well-oriented crypts for each group. The labeling index was calculated by dividing the number of BrdU-positive cells by the total number of nucleated cells for each well-oriented crypt.

To investigate the localization of EB1, DLG1, and DLG5 in the inflamed colon, fluorescent immunochemistry was performed. Anti-CD31 polyclonal antibody (Santa Cruz Biotechnology) and anti-EB1, anti-DLG1, and anti-DLG5 polyclonal antibodies (Abcam) were used as primary antibodies. Alexa Fluor 488–conjugated anti-rabbit IgG antibody and Alexa Fluor 594–conjugated anti-mouse IgG antibody (1:200; Invitrogen, Carlsbad, CA) were used as secondary antibodies. Immunostained sections were visualized using a Biorevo immunofluorescence microscope (Keyence, Osaka, Japan).

Analysis of Colonic Microvasculature Density

Vascular density was calculated using an international consensus method to quantify angiogenesis, as previously described.[19] Briefly, CD31-stained distal colonic sections were scanned and the number of vessels within the mucosa was counted to identify the most vascularized area. Vascular density per field was obtained from at least five microphotographs of the most vascularized mucosa for each rat. Quantitative analysis of the data was performed using WinROOF software version 6.0 (Mitani Corp., Fukui, Fukui, Japan).

Real-Time PCR

Total RNA was isolated from inflamed mucosa of the distal colon 3 cm from the anus, and cDNA was synthesized. Real-time RT-PCR was performed using a Thermal Cycler Dice Real Time System with SYBR Premix Ex TaqII (Takara Bio Inc., Otsu, Shiga, Japan). The primers used were: *Tnfa*, 5′-AACTCGAGTGACAAGCCCGTAG-3′ and 5′-GTACCAC-CAGTTGGTTGTCTTTGA-3′; *Il1b*, 5′-GCTGTGGCAGC-TACCTATGTCTTG-3′ and 5′-AGGTCGTCATCATCCCA-CGAG-3′; *Il10*, 5′-CAGACCCACATGCTCCGAGA-3′ and 5′-CAAGGCTTGGCAACCCAAGTA-3′; *Ptgs2*, 5′-GCGA-CTGTTCCAAACCAGCA-3′ and 5′-TGGGTCGAACTTG-AGTTTGAAGTG-3′; *Ptges*, 5′-TACGCGGTGGCTGTCA-TCA-3′ and 5′-CTCCACATCTGGGTCACTCCTG-3′; *Ppia*, 5′-GGCAAATGCTGGACCAAACAC-3′ and 5′-AAACGC-TCCATGGCTTCCAC-3′. The number of target molecules was normalized against those of *Ppia* as an internal control.[20]

Reporter Gene Assay of Wnt Signaling

To measure Wnt signaling activity, rat embryonic fibroblasts (REFs) were isolated from E12.5 embryos of F344 and KAD rats in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. REFs were then plated on 24-well culture plates 24 hours before transfection. Lipofectamine LTX (Invitrogen) was used to co-transfect REFs with pTOPFLASH or pFOPFLASH vector (Millipore, Billerica, MA) and pSV-β-Gal vector (Promega Corp., Madison, WI) as an internal control according to the manufacturer protocol. Cells in half of the plates were stimulated with 150 ng/mL of Wnt3a (R&D Systems Inc., Minneapolis, MN) 3 hours later. Luciferase activities were measured 24 hours after transfection with Luciferase Assay Systems (Promega).

Isolation of VECs

VECs were isolated from the thoracic aorta of four F344 and four KAD rats aged 8 weeks, as described previously.[21] VECs were cultured in MCDB 131 medium supplemented with epidermal growth factor, endothelial cell growth supplements, vascular endothelial growth factor, hydrocortisone, heparin, and 2% fetal bovine serum. Almost all cultured cells were of endothelial origin, as assessed by staining with Dil-Ac-LDL (Biomedical Technologies Inc., Stoughton, MA). All VECs were examined twice in all *in vitro* experiments to confirm their reproducibility.

Immunofluorescence Microscopy

Cells cultured on collagen-coated coverslips were fixed with 4% paraformaldehyde at 37°C for 15 minutes. After permeabilization and a blocking reaction, they were incubated with antibodies against N-terminal APC (H-290; Santa Cruz), C-terminal APC (C-20; Santa Cruz), α-tubulin (YL1/2; Abcam), paxillin (Abcam), and EB1 (Abcam). They were then treated with Alexa Fluor–conjugated secondary antibodies and phalloidin (Invitrogen), followed by staining with DAPI.

In Vitro Proliferation, Migration, Adhesion, and Tube Formation Assay

A BrdU incorporation assay was performed using the BrdU Labeling and Detection Kit III (Roch Diagnostics GmbH, Mannheim, Germany), according to the manufacturer protocol. Briefly, VECs were plated and labeled with 100 μmol/L BrdU for 6 hours. Cells were stained with the anti–BrdU-POD antibody and quantified by measuring absorbance with an enzyme-linked immunosorbent assay (ELISA) reader. The migration activity of VECs was measured using a wound healing assay, as described

Table 1 Expression Levels of Inflammatory Cytokines in KAD Rats

	Week 0		Week 1		Week 2		Week 4	
Gene	KAD	F344/NSlc	KAD	F344/NSlc	KAD	F344/NSlc	KAD	F344/NSlc
Tnfα	1.26 ± 0.91	1.84 ± 1.53	0.47 ± 1.10	0.25 ± 0.30	88.93 ± 236.33	9.19 ± 11.50	3.24 ± 3.76*	0.22 ± 0.36
Il1β	0.24 ± 0.11	0.49 ± 0.32	118.08 ± 153.51	26.20 ± 33.14	927.33 ± 1204.49	889.79 ± 1233.85	110.61 ± 148.11*	4.70 ± 6.62
Il10	0.02 ± 0.01	0.04 ± 0.03	1.19 ± 1.59	0.26 ± 0.29	2.62 ± 4.37	3.17 ± 2.77	0.65 ± 0.77*	0.05 ± 0.05
Ptgs2/Cox2	0.75 ± 0.37	0.98 ± 0.18	0.34 ± 0.32	0.61 ± 0.77	186.45 ± 344.84	39.82 ± 101.57	13.66 ± 18.92*	0.16 ± 0.23
Ptges	0.62 ± 0.25	0.44 ± 0.46	13.14 ± 21.22	1.11 ± 1.56	73.20 ± 90.45	58.30 ± 77.77	11.49 ± 12.06*	2.18 ± 2.47

*$P < 0.05$.

horseradish peroxidase–conjugated anti-mouse and anti-rabbit IgG (1:2000, Sigma). Immunoblotted proteins were visualized using an ECL select Western blotting detection system (GE Healthcare, Piscataway, NJ).

Statistical Analysis

Student's *t*-test was performed, and SDs were calculated using the statistics package in Microsoft Excel (Microsoft Inc., Redmond, WA). $P < 0.05$ was considered statistically significant.

Results

KAD Rats Display Severe Acute Colitis and Sustained Colonic Inflammation

DSS can effectively induce colonic inflammation in rats, and a loss of body weight, diarrhea, and fecal blood are observed as the clinical symptoms.[24] Loss of body weight was noted from day 8 to day 10 in both F344 and KAD rats; however, there were no differences between the two strains of rat. There were no differences in water and food consumption during exposure to DSS (data not shown). KAD rats obtained higher inflammatory scores, mainly resulting from watery diarrhea and visible fecal blood, compared with F344 rats during exposure to DSS. This finding was true even after terminating exposure to DSS (Figure 1A).

Macroscopically, severe inflammation was generally located in the distal colon of the F344 and KAD rats. Prominent macroscopic features of KAD rat colon were dilation, primarily the distal part of the colon (one-third of the colon from the anus), thickening of the colonic wall, and extensive loss of the mucosa accompanied by bleeding. These findings were also observed in F344 rats, but the severity was less than that of KAD rats. The changes in KAD rats lasted until week 4; in F344 rats they lasted until week 2, and this was associated with their clinical symptoms.

Histopathologic analysis revealed the presence of mucosal ulceration, crypt loss, diffuse inflammatory cell infiltrate of the lamina propria and submucosa, debris, exudates, edema, and congestion and dilatation of the capillary blood vessels in the affected colons of both F344 and KAD rats at weeks 1 and 2 (Figure 1B). Of note, at week 1, eosinophilic structures covering the damaged mucosa were observed in F344 rats, whereas few structures

Table 2 Labeling Index Observed for DSS-Treated Colonic Mucosa

Week	F344	KAD
2	14.1 ± 7.9	16.1 ± 6.7
4	4.1 ± 1.5	8.5 ± 4.0*

The labeling index was calculated by dividing the number of BrdU-positive cells by the total number of nucleated cells per well-oriented crypt.
*$P < 0.001$ compared with F344 rats.

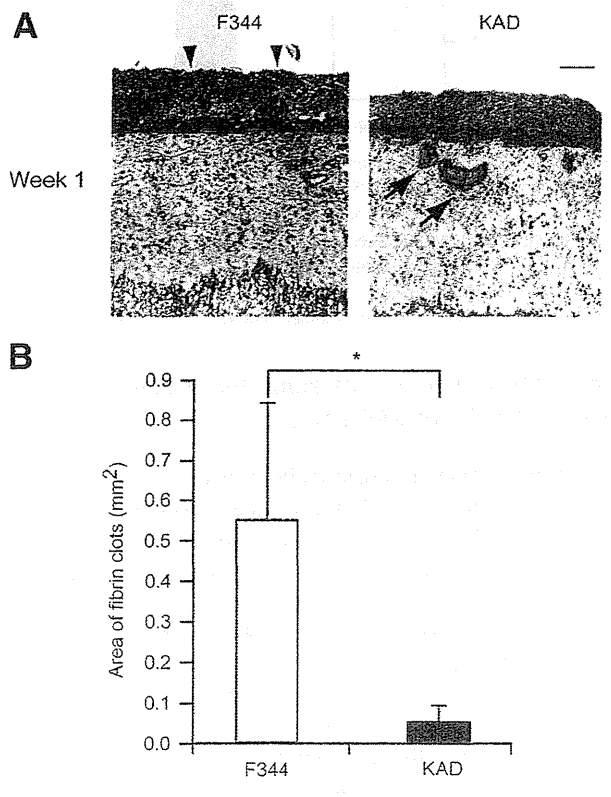

Figure 2 Lack of fibrin clot formation along the mucosal ulcer in the colon of KAD rats. **A:** PTAH-stained sections of F344 and KAD rats at week 1. Fibrin pseudo-membrane covering the surface of the damaged mucosal epithelia was observed in F344 rat colon (**arrowheads**). Fibrin pseudo-membranes were absent on the colonic mucosa of KAD rats; instead, fibrin microthrombi were observed beneath the lamina propria (**arrows**). Scale bar = 100 μm. **B:** The area of fibrin layers covering the surface of DSS-induced inflamed mucosa per rat. *$P < 0.001$.

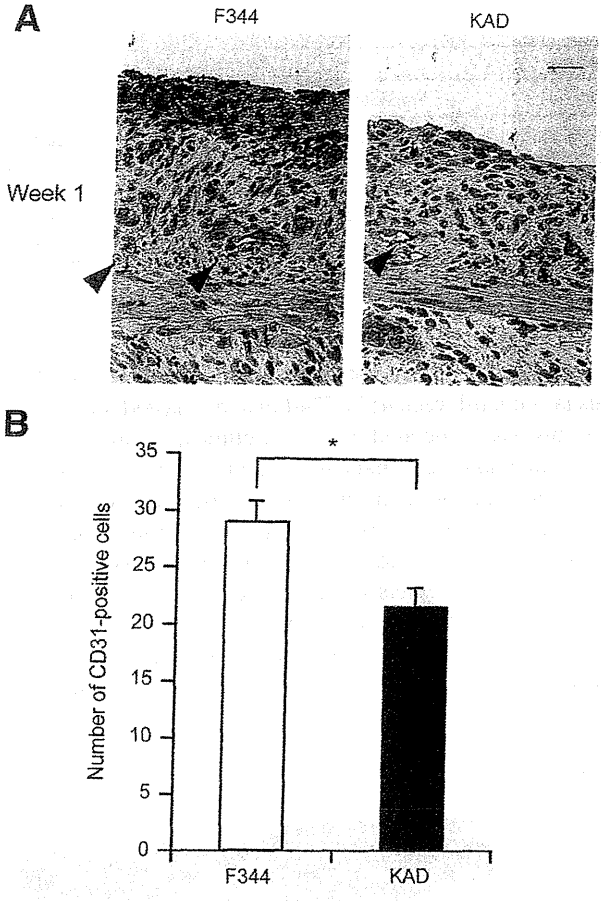

Figure 4 Reduction of angiogenesis associated with an absence of fibrin pseudo-membrane in the DSS-treated colon of KAD rats. **A:** IHC for CD31 of the distal colon of F344 and KAD rats at week 1. CD31-positive cells were clearly present along the ulcerated mucosa of F344 rats, whereas these cells were observed in much smaller numbers in the mucosa of KAD rats (**arrowheads**). Scale bar= 50 μm. **B:** Number of CD31-positive cells per field within the mucosa along the inflamed mucosa. *$P < 0.001$.

The Absence of Fibrin Clots along the Damaged Mucosal Epithelia of KAD Rat Colon

To determine whether the eosinophilic structures that had been observed in the colon of F344 rats at week 1 were fibrin clots, we performed PTAH staining on the colonic tissue sections. For F344 rats, fibrin clots formed a pseudo-membrane covering the surface of the inflamed colonic mucosa (Figure 2A). In contrast, for KAD rats, formation of the pseudo-membrane consisting of fibrin clots was much less. Instead, fibrin was deposited mostly in microvessels immediately below the lamina propria and formed microthrombi. The area of the fibrin clots that covered the inflamed mucosa was significantly higher for F344 rats than for KAD rats (0.56 ± 0.29 mm^2 versus 0.057 ± 0.039 mm^2; $P < 0.001$) (Figure 2B). The area of fibrin microthrombi was significantly greater for KAD rats than for F344 rats (0.060 ± 0.048 mm^2 versus 0.0047 ± 0.0050 mm^2; $P < 0.04$). These results indicate that the formation of fibrin layers was defective over the damaged mucosa of KAD rats. Thus, the damaged mucosa of KAD rats has low potential to form fibrin layers, which make an important contribution to healing the eroded mucosa.

APC Is Expressed in the VECs in the Inflamed Colon

To find a potential association of the presence of APC with colitis, the expression of APC protein was examined in rat colonic tissue. Sequential change of APC expression was generally common in both F344 and KAD rats. At week 0, APC protein was expressed in the cell membrane and cytoplasm of normal mucosal epithelial cells (Figure 3A). At week 1, immediately after DSS treatment, APC protein was highly expressed in VECs in the inflamed submucosa, although no APC could be detected at the mucosal epithelial cells because of their disruption by severe ulceration. At week 4, the expression of APC had recovered in regenerative mucosal epithelial cells and VECs. We confirmed that APC-positive cells in the inflamed distal colon were VECs by immunostaining of serial sections using the anti-CD31 antibody, a positive marker for VECs (Figure 3B). These findings indicate that the expression of APC protein was induced in VECs of the submucosa when colon inflammation occurred.

Reduced Angiogenesis With Inflamed Colonic Mucosa in KAD Rats

Given that APC protein functions in VECs, we examined microvessel angiogenesis in the damaged mucosa by measuring the number of CD31-positive cells in the damaged colonic mucosa at week 1. For F344 rats, many CD31-positive cells were present along the mucosa, which was often associated with the presence of fibrin-like clots (Figure 4A). For KAD rats, fewer CD31-positive cells were observed. The number of microvessels per field along the inflamed mucosa of KAD rats was significantly lower than that of F344 rats (21.70 ± 1.59 versus 29.10 ± 1.83; $P < 0.001$) (Figure 4B). These results indicate that microvessel angiogenesis for healing was reduced in the damaged mucosa of KAD rats compared with F344 rats.

Truncated APC of KAD Rats Does Not Affect Wnt Signaling

To clarify whether a loss of the C terminus of APC could affect the regulation of Wnt signaling, we cultured REFs from E12.5 embryos of F344 and KAD rats. We then tested these REFs for their ability to inhibit Tcf-regulated transcription in transfection assays. TOPFLASH luciferase activity in the REFs from F344 and KAD rats was similar to control FOPFLASH activity. Luciferase activity in the REFs of both F344 and KAD rats increased with Wnt3a treatment (Supplemental Figure S1). These results indicate that truncated APC of KAD rats can normally regulate the Tcf-regulated transcription through a Wnt ligand.

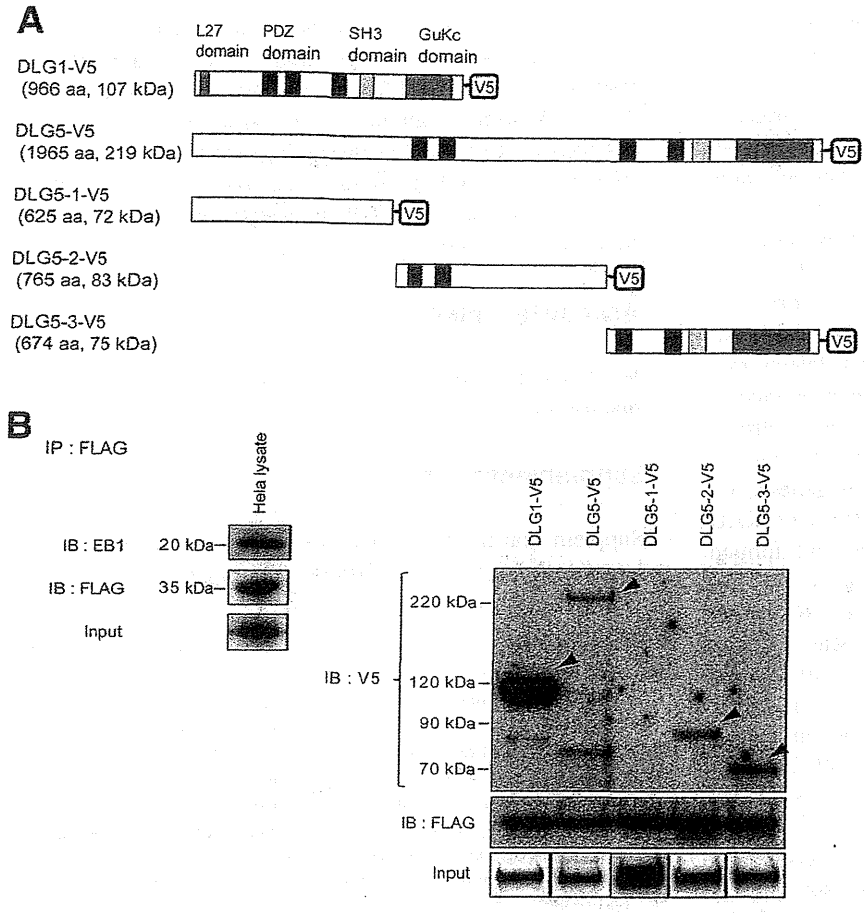

Figure 6 Association of the APC C terminus with EB1, DLG, and DLG5 *in vitro*. **A:** Domain structures and schematic representation of the DLG1 and DLG5 constructs encoding an N-terminal V5 epitope tag: DLG1-V5 (966 amino acids; 107 kDa), DLG5-V5 (1965 amino acids; 219 kDa), DLG5-1-V5 (625 amino acids; 72 kDa), DLG5-2-V5 (765 amino acids; 83 kDa), and DLG5-3-V5 (674 amino acids; 75 kDa). **B:** Binding of the EB1, DLG1, and DLG5 proteins to the FLAG-tagged C terminus of APC (FLAG-APC-C-term; 347 amino acids, 37 kDa) that is absent in the KAD rat. The FLAG-APC-C-term and each construct were co-transfected to Hela cells. The FLAG-APC-C-term was then immunoprecipitated by the FLAG antibody. Immunoprecipitated proteins were subjected to Western blotting to detect proteins that were co-immunoprecipitated with FLAG-APC-C-term. EB1 was detected using the anti-EB1 antibody. DLG1, DLG5, DLG5-1, DLG5-2, and DLG5-3 were detected using the anti-V5 antibody. EB1, DLG1, DLG5, DLG5-2, and DLG5-3 were co-immunoprecipitated with the FLAG-APC-C-term (**arrowheads**).

cytoskeleton. APC protein accumulated at the ends of the MTs at the migrating edges of both F344 and KAD rat VECs (Supplemental Figure S2). The incorporation of BrdU into KAD rat VECs was not different from that seen in F344 rat VECs (F344: 1.00 ± 0.04 versus KAD: 1.01 ± 0.03; $P = 0.92$). A wound healing assay demonstrated that the migration activity of KAD rat VECs stimulated with vascular endothelial growth factor was not different from that of F344 rat VECs (F344: 44.9 ± 6.03 versus KAD: 41.7 ± 7.30; $P = 0.11$).

A wash assay revealed significantly higher adhesion activity in KAD rat VECs than that in F344 rat VECs (Figure 5A). Immunostaining with antipaxillin antibody demonstrated that the number of focal adhesion sites of KAD rat VECs was significantly higher than that of F344 rat VECs (Figure 5, B and C). The capillary-like structure of KAD rat VECs induced on Matrigel revealed apparent differences in morphology compared with those of F344 rat VECs (Figure 5D). The length of tubes of KAD rat VECs was significantly shorter than that of F344 rat VECs (Figure 5E). The number of branches of KAD rat VECs was significantly fewer than that of F344 rat VECs (Figure 5F). These findings indicate that KAD rat VECs had normal physiologic function in morphology, proliferation, and migration but defects in adhesion and tube formation.

EB1 and DLG5 Could Bind to the C Terminus of APC and Are Expressed in the VECs of the Inflamed Colonic Region

To find molecules that interact with the C terminus of APC at the VEC in the inflamed area, we performed an Immunoprecipitation assay and fluorescent IHC. The C terminus of APC, which is absent in KAD rats, can interact with EB1 and DLG1.[12] Recently, DLG5 has been reported to be associated with pathogenesis of IBD.[27] Because DLG5 and DLG1 share the postsynaptic density protein-95/disks large/zonula occludens-1 (PDZ) domains that are known to bind to the C terminus of APC (Figure 6A) and the most characteristic feature of IBD is sustained inflammation of the colon, we examined whether DLG5, similar to EB1 and DLG1, can bind to the C terminus of APC.

Similar to EB1 and DLG1, full-length DLG5 was co-immunoprecipitated with the FLAG-APC-C-term (Figure 6B). Although we could not detect DLG5-1-V5, containing the first third of DLG5, we could detect DLG5-2-V5 and DLG5-3-V5, containing the second and final third of DLG5, respectively (Figure 6B). Both DLG5-2-V5 and DLG5-3-V5 contain PDZ domains, similar to DLG1, but the DLG-1-V5 contained no PDZ domain. Thus, these results indicate that the C terminus of APC could interact with the PDZ domain of DLG5.

higher incidences and multiplicities of colon tumors found in KAD rats compared with the control F344 rats.[16]

Immediately after DSS treatment, KAD rats had an abnormal deposition of fibrin in microvessels, instead of a normal formation of fibrin layers to cover the damaged mucosa. KAD rats also had decreased microvessel angiogenesis in the damaged colonic mucosa immediately after DSS treatment. Given the evident increase in expression of APC in VECs of the edematous submucosa, it is likely that APC has a critical role in angiogenesis that is driven in response to acute inflammation of the colon and that KAD rats have defects in such angiogenesis. Such defects may result in a delay in the repair process of the damaged mucosa possibly because of the shortage of oxygen and nutrition supply and therefore a persistence of colitis in KAD rats.

To determine how APC is involved in angiogenesis, we characterized the physiologic function of VECs of KAD rats. KAD rat VECs had stronger adhesion and formed abnormal tube morphology, which was characterized by a smaller number of branching points and a shorter length when VECs were stimulated to form tubes on Matrigel. The adhesion activity of VECs plays an important role in tube formation.[33,34] Therefore, it is likely that the enhanced adhesion activity may result in the low branched and short tubes found in KAD rat VECs. Although VECs were prepared from the thoracic aorta in this study, defects in tube formation may explain the decreased number of microvessels found in the damaged colonic mucosa of KAD rats.

Expression of both APC and DLG5 was specifically induced in the VEC of the inflamed colon at week 1, immediately after DSS treatment. DLG5 interacted with the C terminus of APC, probably via its PDZ domains. The DLG protein family interacts with transmembrane proteins in endothelial cells and is associated with angiogenesis.[35] Thus, we consider that the APC-DLG5 molecular pathway may function in the regulation of angiogenesis and cell adhesion of VECs.

It is known that abnormal angiogenesis contributes to the initiation and perpetuation of IBD and that microvessel dysfunction causes poor mucosal healing in IBD.[36,37] Similar to IBD patients, KAD rats had reduced angiogenesis in the inflamed mucosa and the KAD VECs had reduced adhesion activity. The human *DLG5* locus is associated with the pathogenesis of IBD.[27] In rats, expression of DLG5 can be induced by DSS treatment, and DLG5 binds to the C terminus of APC, which is absent in KAD rats. These results suggest that both APC and DLG5 may be involved in the pathogenesis of IBD. Further genetic analysis of relevant human populations may clarify the involvement of APC in the development of colitis.

It is expected that mutant APC derived from the $Apc^{\Delta 2523}$ allele may coexist with wild-type APC in the heterozygous KAD ($Apc^{\Delta 2523}/+$) rat because transcripts from the mutant allele can escape nonsense-mediated decay. Thus, further study using heterozygous rats may reveal dominant negative functions of the mutant APC.

In summary, we found a new function of APC in the pathogenesis of colonic inflammation, which appears to be mediated by the regulation of cellular adhesion activity of VECs. Molecular interaction of APC with DLG5 may function in the regulation of angiogenesis and cell adhesion of VECs. Our findings suggest that APC may contribute to the pathogenesis of IBD, in which patients are more susceptible to colorectal cancers.

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Supplemental Data

Supplemental material for this manuscript can be found at *http://dx.doi.org/10.1016/j.ajpath.2012.12.005.*

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