5-Aminosalicylic Acid Mediates Expression of Cyclooxygenase-2 and 15-Hydroxyprostaglandin Dehydrogenase to Suppress Colorectal Tumorigenesis

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Abstract. *Background: Cyclooxygenase-2 (COX-2) is a key enzyme that produces prostaglandin E2 (PGE2) and plays an important role in colorectal tumor growth. In addition, recent researches focused on 15-hydroxyprostaglandin dehydrogenase (15-PGDH), which degrades PGE2. Here we determined the effect of 5-aminosalicylic acid (5-ASA) on COX-2 and 15-PGDH expression and investigated its preventive effect for colorectal cancer (CRC). Materials and Methods: HT-29 cells were used in the in vitro experiments. c-Ha-ras transgenic mice were employed in order to explore the chemopreventive effects. Western blotting analysis was performed and the protein expression of COX-2 and 15-PGDH was quantified. Results: 5-ASA significantly suppressed COX-2 expression and induced 15-PGDH expression in HT-29 cells. In the transgenic mice, oral 5-ASA intake reduced the incidence of colorectal tumor formation and the tumor size. Furthermore, we observed a down-regulation of COX-2 and an up-regulation of 15-PGDH in the tissue from colons of these mice. Conclusion: 5-ASA exerts a preventive effect against colorectal tumor development through mediation of COX-2 and 15-PGDH expression.*

Colorectal cancer (CRC) is one of the most common malignant diseases in the world. Despite progresses in therapeutic modalities, advanced CRC is still life-threatening, further highlighting the importance of disease prevention. It has been reported that increased levels of cyclooxygenase-2 (COX-2) are observed in 50% of colorectal adenomas and around 86% of sporadic CRC (1, 2). COX-2 mediates the production of prostaglandin E2 (PGE2) in epithelial tissues, which promotes cell proliferation and inhibits cell death (3, 4). Furthermore, several randomized trials have shown that nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, and selective COX-2 inhibitors, such as celecoxib and rofecoxib, are effective for the prevention of colorectal adenoma (5-9). However, due to possible adverse events, the afore-mentioned medications are not routinely recommended for the prevention of colorectal cancer; Aspirin is known to increase the risk of gastrointestinal (GI) tract bleeding, whereas celecoxib and rofecoxib have been shown to be associated with increased cardiovascular events (10, 11).

CRC is also a serious complication of inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD). A meta-analysis showed that one in five patients with UC will develop CRC over 30 years, with the risk of CRC being influenced by the extent of lesion and duration of the disease (12). Another large population-based study revealed that patients with CD also have an increased risk of CRC, with similar rates of CRC in UC and CD patients (13). Several studies have reported that 5-aminosalicylic acid (5-ASA), the most commonly used anti-inflammatory medication for IBD, is associated with variable reduction in the risk of CRC (14, 15), although some conflicting reports challenge these data (16, 17). It has been suggested that the effect of 5-ASA therapy on reducing the risk of CRC could be mediated by a reduction in mucosal inflammation. On the other hand, some studies have shown that 5-ASA has an antitumor effect, regardless of the persistence of inflammation (18-21), and one previous study reported that 5-ASA down-regulated COX-2 expression and inhibited the proliferation of human colon cancer cells *in vitro*, in part by a COX-2-dependent mechanism (22). These findings suggest the potentiality of 5-ASA as a much safer preventive agent against CRC due to its demonstrated long-term safety in the therapy of IBD.

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In addition to inhibition of COX-2 activity, a recent study showed that the adenoma prevention activity of celecoxib requires the concomitant expression of 15-hydroxy-prostaglandin dehydrogenase (15-PGDH) (23), which is a prostaglandin-degrading enzyme and a physiological antagonist of COX-2. It has been reported that expression of 15-PGDH is high in normal colonic epithelium but is diminished in cancer tissues (24). Furthermore, a previous study demonstrated that several NSAIDs induced 15-PGDH expression in the human colon cancer cell line, HT-29 (25).

In this study, we investigated the role of 5-ASA treatment in HT-29 cells and in a mouse model, on COX-2 and 15-PGDH.

Materials and Methods

Cell culture. HT-29 cells were cultured in McCoy's 5A modified medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.1 mM minimum essential medium (MEM) non-essential amino acid solution, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Life Technologies Corp., Carlsbad, CA, USA) at 37˚C in a humidified atmosphere of 95% air and 5% CO2. 5-ASA (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in the culture medium immediately before use and the pH of the solution was adjusted to 7.4 with NaOH. To examine whether 5-ASA affects the expression of COX-2 and 15-PGDH, HT-29 cells were plated into 90 mm culture dishes, and after 24 hours, cells were cultured in the absence or presence of 5-ASA (1, 5, or 10 mM) for 48 h.

Mice. C57BL/6J (wild-type; WT) mice were purchased from CLEA Japan, Inc (Tokyo, Japan), and were assigned to the WT group. c-Ha-ras transgenic mice of C57BL/6J background were a kind gift from Dr. Tatsuji Nomura (Central Institute for Experimental Animals, Kawasaki, Japan). The original characteristics of the transgenic mice are described elsewhere (26, 27). The transgenic mice were randomized to Ras or Ras+5ASA group. The WT and the Ras group consisted of four males and six females respectively. Six males and six females belonged to the Ras+5ASA group. Body weight of mice was measured every week. All mice were raised in specific-pathogen free (SPF) conditions.

1,2-Dimethylhydrazine dihydrochloride (DMH) and 5-ASA treatments. DMH (Sigma-Aldrich) was suspended in phosphate-buffered saline (pH 7.0) at a concentration of 4 mg/ml and subcutaneously injected into all mice at a dose of 20 mg/kg body weight, according to a previous study (26). DMH administration was initiated at 11 weeks of age, and mice were injected once a week for 20 weeks. In addition, 11-week-old mice assigned to the Ras+5-ASA group were treated orally with 5-ASA, which was mixed with powdered CLEA Rodent Diet CE-2 (CLEA Japan, Inc, Tokyo, Japan), until termination of the study. The dose of 5-ASA was determined by the formula for dose translation based on the body surface area (28). This calculation resulted in the dose of 820 mg/kg/day in a mouse, which equates to a 4000 mg/day dose of 5-ASA for a 60 kg person. Mice in the WT and the Ras group were fed powdered diet without 5-ASA. All mice were sacrificed at 35 weeks of age (Figure 1).

Histopathology. The large intestine (from the cecum to the anus) of the sacrificed mice was isolated, and the length was measured in a relaxed position without stretching. They were opened longitudinally and cleaned in normal saline, and the tumor lesions were macroscopically examined under a dissecting microscope with indigo carmine. The tissues were fixed in 10% neutral-buffered formalin solution (Wako, Osaka, Japan) and embedded in paraffin. The specimens were stained with hematoxylin and eosin (H&E) according to standard procedures.

Immunohistochemistry. Paraffin sections were deparaffinized following a previously described protocol (29). After deparaffinization, the sections were incubated with the rabbit polyclonal antibodies to COX-2 (1:500) or to prostaglandin dehydrogenase 1 (1:200) at 4˚C overnight in a moist chamber. After the primary reaction, the sections were washed twice with PBS, incubated with peroxidase-conjugated goat anti-rabbit IgG polyclonal antibody (Nichirei, Tokyo, Japan) at room temperature for 30 minutes, developed with 3,3'-diaminobenzidine and H_2O_2 in PBS (Nichirei) for 9 minutes or 13.5 minutes, for COX-2 and 15-PGDH respectively, and counterstained with hematoxylin. As a positive control for 15-PGDH, WT mice without DMH treatment were simultaneously evaluated.

Protein extraction and western blot analysis. Total protein was extracted from treated HT-29 cells using Complete Lysis-M, EDTA-free (Roche Diagnostics, Indianapolis, IN, USA) and from mouse colons using the T-PER Tissue Protein Extraction Reagent (Thermo Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Proteins were separated on 7.5% Mini-PROTEAN TGX Gels (Bio-Rad Laboratories, Hercules, CA, USA). The iBlot Gel Transfer Dvice, iBlot PVDF Transfer Stack and iBlot Western Detection Kit (Invitrogen) were employed to transfer proteins onto polyvinylidene fluoride (PVDF) membranes, block the blots, and incubate with primary and secondary antibodies, as per the manufacturer's protocol. Rabbit polyclonal antibodies to COX-2 (ab15191; 1:100) and prostaglandin dehydrogenase 1 (ab37148; 1:500) (Abcam, Cambridge, UK), the latter of which was used to detect 15-PGDH, were used as primary antibodies. After analysis of COX-2 and 15-PGDH, each blot was incubated with a rabbit polyclonal antibody to beta-actin (ab8227; 1:500) (Abcam) to ascertain equivalent loading of the lanes. The immunoreactive bands were detected, and their intensities were analyzed using the ImageQuant LAS 4000 mini system (GE Healthcare UK Ltd, Buckinghamshire, UK).

Statistical analysis. Statistical analyses of the protein expression of COX-2 and 15-PGDH, length of the large intesitine, tumor diameter or incidence of colorectal tumors were performed with the Student's *t*-test, the Welch's *t*-test or the Fisher exact test using the IBM SPSS Statistics version 18 (SPSS, Chicago, IL, USA). Statistical significance was defined as $p<0.05$.

Results

The effect of 5-ASA on COX-2 and 15-PGDH expression in HT-29 cells. We first examined the effect of 5-ASA on the protein expression of COX-2 and 15-PGDH, which have been reported to play important roles in the growth or

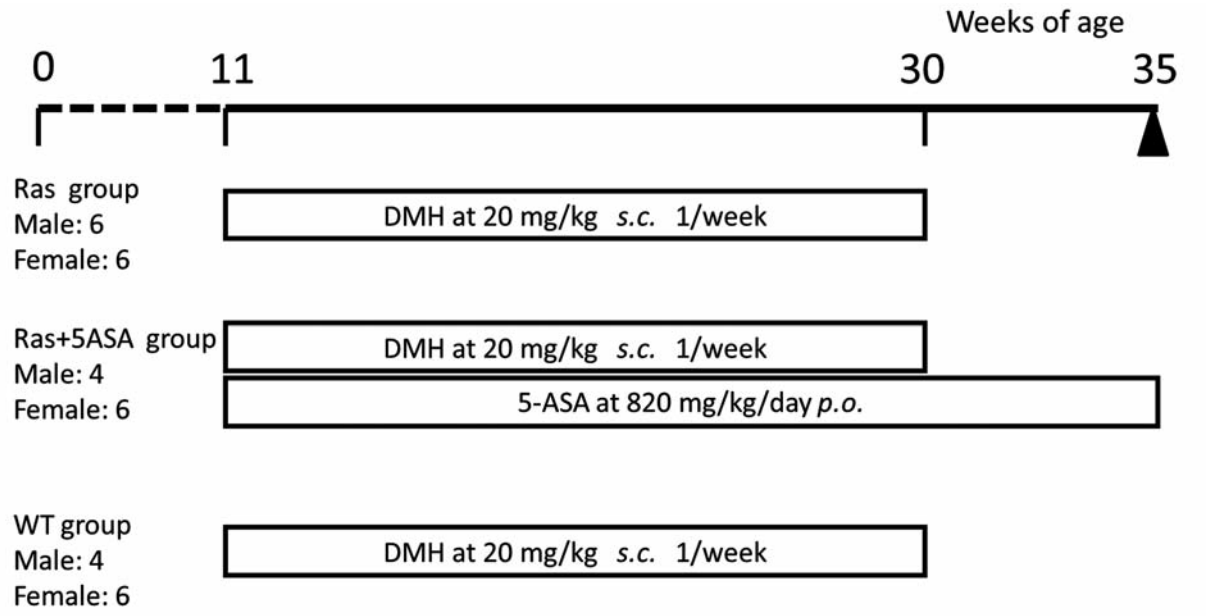

Figure 1. *Experimental protocol of 1,2-dimethylhydrazine dihydrochloride (DMH) and 5-aminosalicylic acid (5-ASA) treatments. Wild-type (WT) mice and c-Ha-ras transgenic mice were injected with DMH (20 mg/kg) subcutaneously once a week for 20 weeks from 11 weeks of age. In addition, mice in the Ras+5ASA group were orally administered 5-ASA (820 mg/kg/day) every day from 11 weeks of age until the end of the study period. Mice were sacrificed at 35 weeks of age.*

suppression of colorectal tumors via regulation of PGE2 (23, 24, 30, 31), in HT-29 cells. After treatment for 48 hours, western blotting analysis was performed for COX-2 and 15-PGDH, and the expression of these proteins were quantified and normalized by beta-actin expression. In HT-29 cells treated with 1, 5, or 10 mM 5-ASA, COX-2 expression was remarkably reduced in a concentration-dependent manner ($p<0.01$) (Figure 2A and B). By contrast, 5-ASA significantly up-regulated 15-PGDH ($p<0.01$, $p=0.040$, and $p=0.032$, respectively), and the effect did not differ between the concentrations used (Figure 2C and D). The effect of 5-ASA on induction of 15-PGDH appeared to be at the same plateau even at the lowest concentration (1 mM).

The effect of DMH and 5-ASA treatments on murine colon. In accordance with a previous study, c-Ha-ras transgenic mice treated with DMH developed sporadic colorectal tumors. There was no macroscopic evidence of colonic inflammation. Representative microscopic images of a non-tumorous area and neoplastic lesions are shown in Figure 3A-F. Histopathological evaluation revealed no significant inflammatory infiltrate in the background mucosa, and the presence of nodular and villous tumors with several degrees of dysplasia. The gross morphology of the large intestines taken from animals from the WT, Ras and Ras+5-ASA groups were not different (Figure 3G). The average lengths of large intestines were 62.4±2.7 mm, 60.1±2.4 mm and 61.5±3.5 mm in the WT, Ras and Ras+5-ASA groups, respectively, with no significant difference among these groups (Figure 3H). Each large intestine was opened longitudinally in order to examine the tumor lesions (Figure 3I).

Tumor-preventive effect of 5-ASA in c-Ha-ras transgenic mice. We examined the number and the size of tumors in colonic specimens obtained from the mice. The incidence of colorectal tumors was 20% in WT (2/10), 83% in Ras (10/12) and 40% in Ras+5ASA (4/10) groups. The incidence in the Ras+5ASA group was significantly lower than that in the Ras group ($p=0.048$) (Figure 4A). The longest tumor diameter was 1.0 mm, 1.9±1.1 mm and 1.1±0.2 mm in the WT, Ras and Ras+5-ASA groups, respectively, which demonstrated that tumors in the Ras+5-ASA group were remarkably smaller than those in the Ras group ($p=0.015$) (Figure 4B). The number, distribution, location and the size of tumors in each group are presented in Figure 4C.

The expression of COX-2 and 15-PGDH in murine colon. In order to investigate the involvement of COX-2 and 15-PGDH expression in murine tumors, we performed immunohistochemistry for COX-2 and 15-PGDH in each group. Firstly, the presence of tumors was confirmed by histopathological analysis of H&E-stained samples; representative images are shown in Figure 5A-C. We detected strong expression of COX-

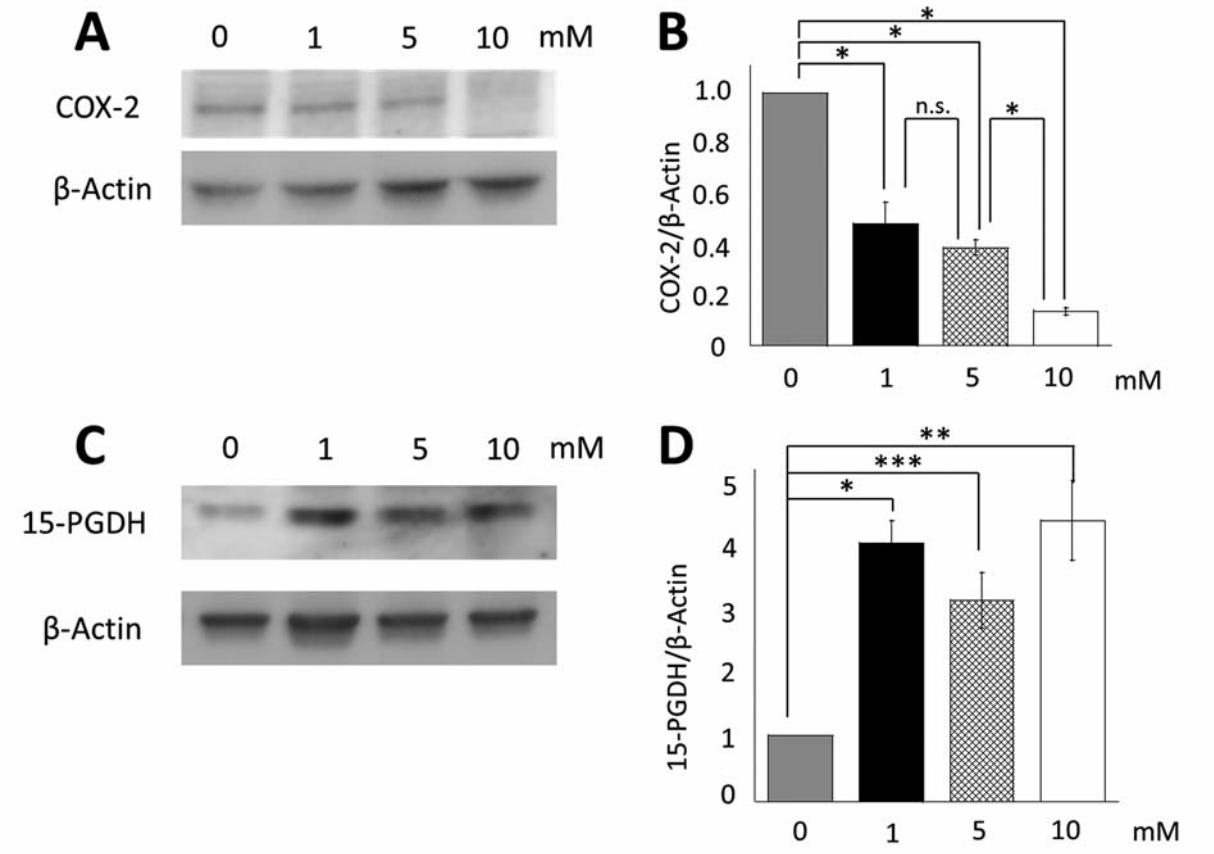

Figure 2. *The effect of 5-aminosalicylic acid (5-ASA) on cyclooxygenase-2 (COX-2) and 15-hydroxyprostaglandin dehydrogenase (15-PGDH) expression in HT-29 cells. HT-29 cells were cultured in the absence or presence of 5-ASA (1, 5, or 10 mM) for 48 hours. Western blotting and quantitative analysis of COX-2, which was normalized to beta-actin protein, were performed. 5-ASA remarkably reduced COX-2 expression (*p<0.01) (A and B) and significantly increased 15-PGDH expression (*p<0.01, **p=0.032, ***p=0.040) (C and D). Representative western blots are shown. Values are expressed as a ratio to the average values of non-treated cells and are the mean±S.E.M. of three separate experiments.*

2 in tumor lesions from all groups (Figure 5D-F). The expression of 15-PGDH was remarkably weak in all groups, regardless of colonic area (Figure 5G-I), as compared to the expression in WT mouse which were not treated with DMH (Figure 5J).

The effect of 5-ASA on COX-2 and 15-PGDH expression in murine colon. In order to determine the mechanism by which 5-ASA exerts tumor preventive effects, we examined the areas without tumors from the Ras group and Ras+5-ASA groups. Assessment of H&E-stained specimens did not show any difference between the two groups (Figure 6A and B), although there appeared to be a tendency for stronger COX-2 (Figure 6C and D) and weaker 15-PGDH expression in the Ras group as compared to the Ras+5-ASA group (Figure 6E and F). Western blot analysis on proteins extracted from the non-tumorous area of the murine colons showed substantial reduction in COX-2 levels and a significant increase in 15-PGDH expression in the Ras+5-ASA animals as compared to the Ras group (p=0.017 and p=0.048, respectively) (Figure 6G-I).

Discussion

Previous studies showed that several growth factor pathways are activated during colonic neoplasia. The activation of prostaglandin signaling is an early and critical step in the development of an adenoma and can be induced by inflammation or mitogen-associated up-regulation of COX-2 (32). The involvement of the COX-2/PGE2 pathway in tumor development and maintenance has also been suggested by the effectiveness of NSAIDs and the selective COX-2 inhibitor, in reducing the incidence and progression of colorectal tumors in animal models and human cancer patients (33). These findings imply that an agent that can suppress COX-2 safely without conferring adverse reactions may become an option for chemoprevention.

In order to address the potential of 5-ASA as a preventive modality for CRC, we first confirmed that 5-ASA can dramatically suppress the expression of COX-2 protein in HT-29 cells even at low concentrations, less than 10 mM. Since it

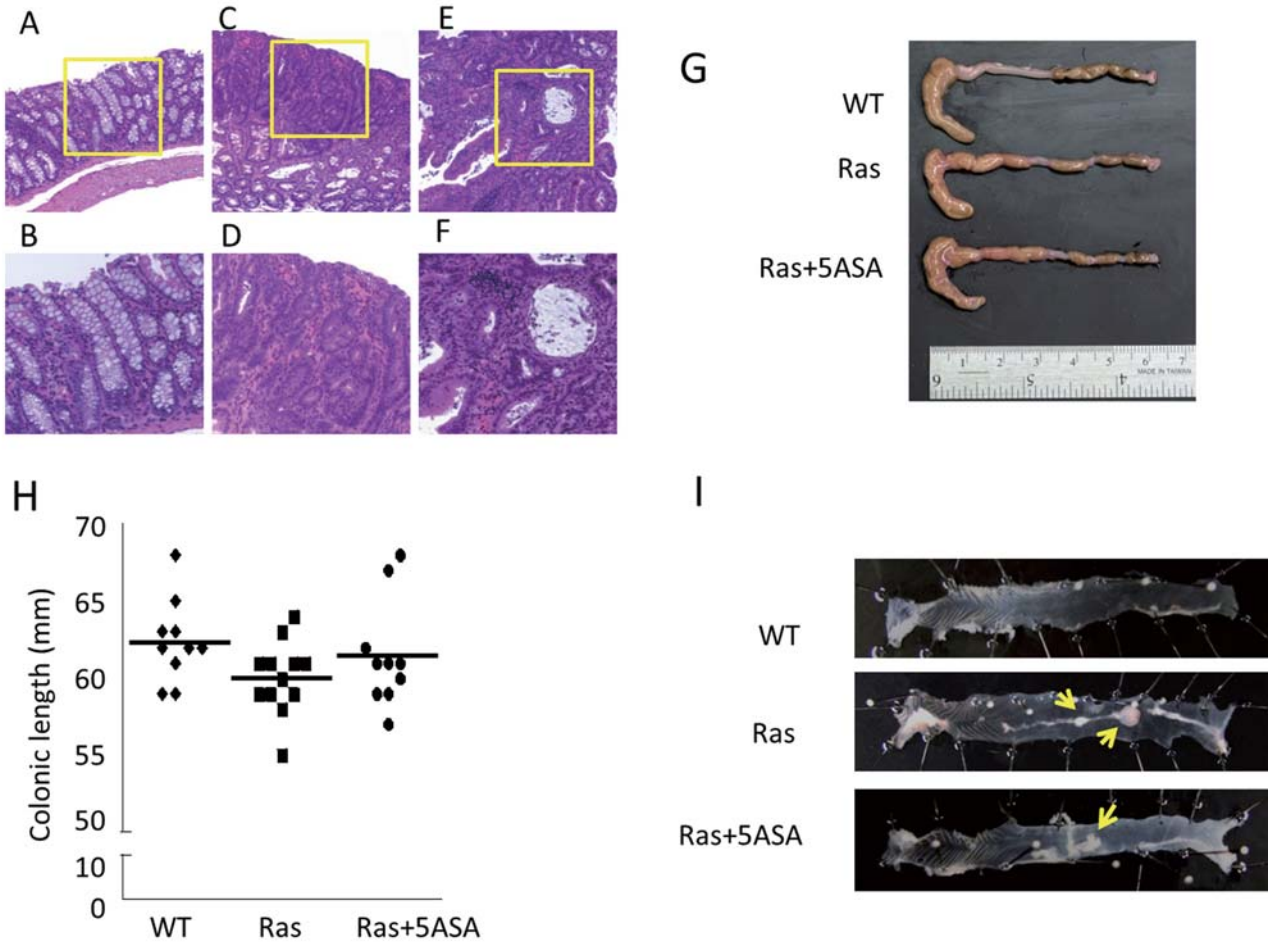

Figure 3. *The characteristics of colon portions taken from a c-Ha-ras transgenic mouse treated with 1,2-dimethylhydrazine dihydrochloride. Representative sections, which were stained with H&E, showing a non-tumorous area and neoplastic lesions of the Ras group are shown at a magnification of ×100 (A, C and E) and ×200 (B, D and F; inset of A, C and E, respectively). Infiltration of inflammatory cells was not detected in the non-tumorous background mucosa (A and B). Colorectal tumors showed nodular or villous growth with several degrees of dysplasia (C-F). The macroscopic appearance, including the length of colons, was not different among WT, Ras and Ras+5-ASA groups (G and H). Each large intestine was opened longitudinally to examine the tumor lesions, which are indicated by arrows (I). Values are mean±S.E.M.*

has been shown that the concentration of 5-ASA in colonic mucosa of UC patients with appropriate therapy is well above 10 mM (34), our results suggest that 5-ASA can down-regulate COX-2 at a concentration that is well within safety limits. On the other hand, colonic adenomas in a significant proportion of individuals are resistant to COX-2 inhibitory treatment (7-9). To explore this mechanism, Yan *et al*. analyzed the role of 15-PGDH, which is a key prostaglandin catabolic enzyme that also acts as a tumor suppressor (23). They examined human rectal mucosa obtained from the Adenoma Prevention with Celecoxib trial (8) and showed that individuals with low colonic 15-PGDH levels exhibited celecoxib resistance and developed new adenomas while receiving the treatment. This study proposed that an agent which increases 15-PGDH expression may be useful for chemoprevention. Furthermore, Chi *et al*. reported that certain NSAIDs are capable of up-regulating 15-PGDH in human colon cancer HT-29 cells (25). Based on these findings, we examined whether 5-ASA has the potential to increase the 15-PGDH protein expression and found that 5-ASA produced the expected effect in HT-29 cells even at an extremely low concentration (1 mM). As far as we know, this is the first report to show the ability of 5-ASA to induce the 15-PGDH protein expression. These *in vitro* results provide evidence regarding the potential of 5-ASA as a chemopreventive agent against colorectal tumor development.

To investigate the preventive effect of 5-ASA *in vivo*, we used c-Ha-ras transgenic mice, in which colorectal tumors develop at an extremely high rate following injection of DMH (26), an agent that has been widely used as a colon-

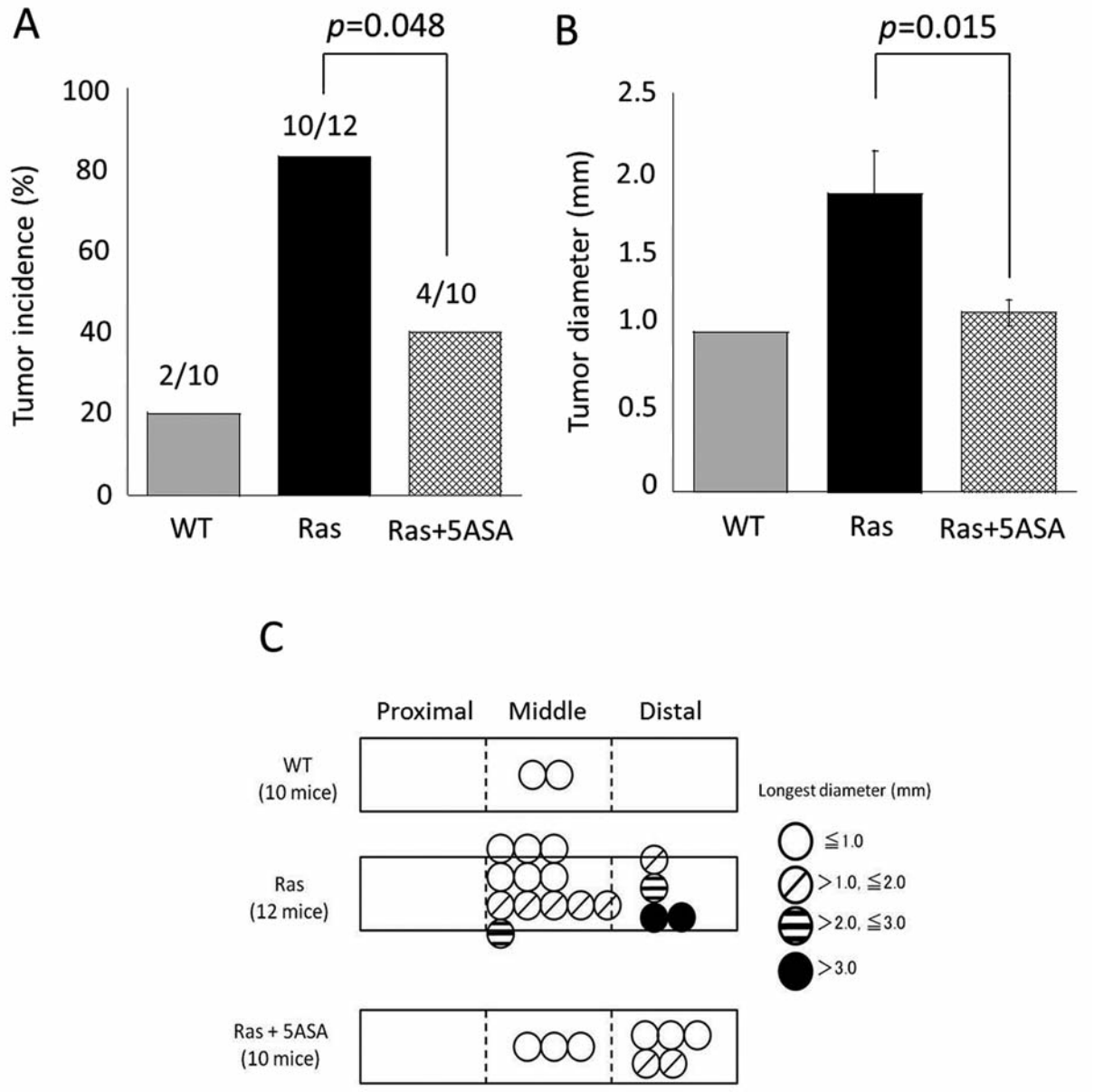

Figure 4. *Tumor preventive effect of 5-aminosalicylic acid (5-ASA) in c-Ha-ras transgenic mice. The incidence of tumor formation in the Ras+5ASA group was significantly lower than that in the Ras group (p=0.048) (A). The greatest tumor diameter in the Ras+5-ASA group was remarkably smaller than that in the Ras group (p=0.015) (B). The number, distribution, location and size of tumors in each group are shown (C).Values are mean±S.E.M.*

selective carcinogen to induce colorectal tumors due to the production of similar histopathological features to human sporadic colorectal tumors (35), although the molecular mechanisms underlying this induction have yet to be revealed. We first confirmed that tumors in the transgenic mouse model were sporadic and there was no inflammation in the background mucosa. We believe that this aspect of the model is highly advantageous for assessing the antitumor effect of 5-ASA because the antitumor and anti-inflammatory effects are largely indistinguishable in common murine tumor models which develop colitis-associated cancer. In this study, we clearly showed that 5-ASA treatment in the Ras+5-ASA group reduced the incidence (p=0.048) and the size (p=0.015) of colorectal tumors in comparison with the Ras group. To explore the mechanisms of this tumor-inhibiting effect of 5-ASA, examination of the expression levels of COX-2 and 15-PGDH in murine colon tissues was performed. By

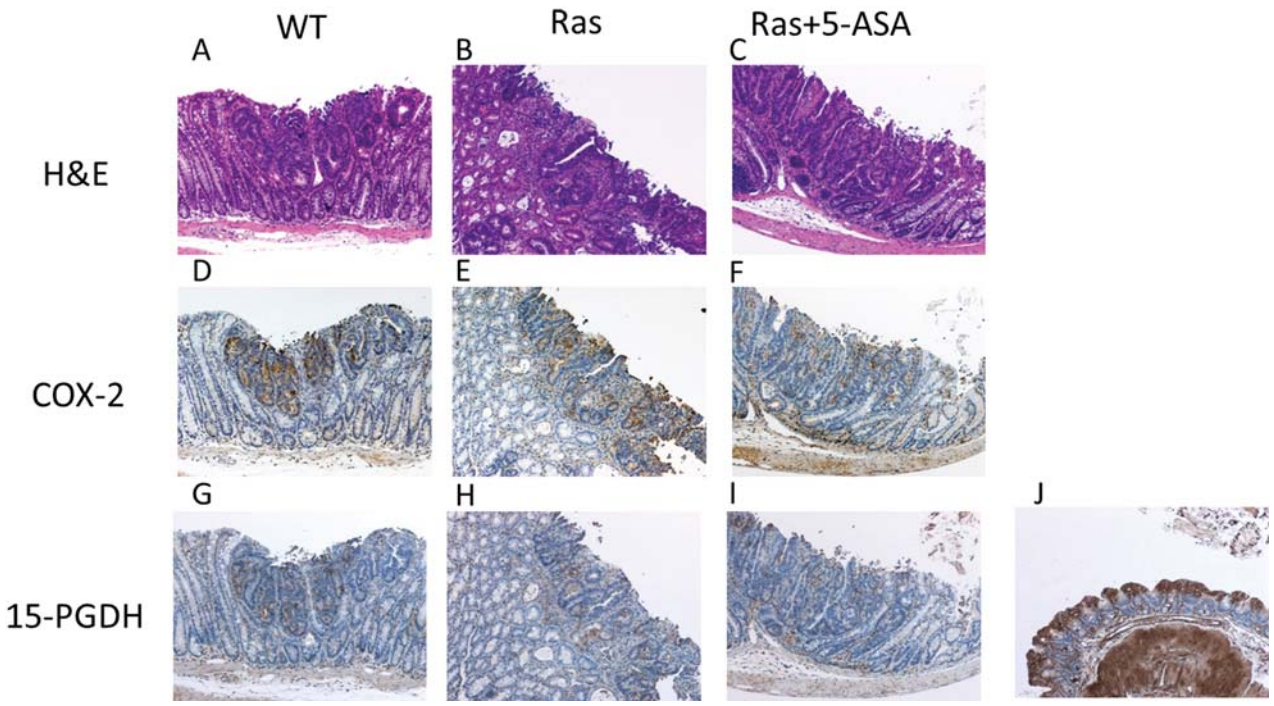

Figure 5. *Immunohistochemistry of tumor specimens. Colonic tumor sections were obtained from WT, Ras and Ras+5-ASA animals, and the presence of neoplastic tissues was confirmed by analysis of H&E stained samples (A-C). Specimens were stained for cyclooxygenase-2 (COX-2) and 15-hydroxyprostaglandin dehydrogenase (15-PGDH). Tumors expressed COX-2 more strongly than non-tumorous area in every group (D-F). The expression of 15-PGDH was weak in all specimens in every group (G-I), as compared to WT (C58BL/6J) mice without 1,2-dimethylhydrazine dihydrochloride (DMH) treatment (J). Representative sections are shown at ×100 magnification.*

immunohistochemistry, we demonstrated that tumors overexpressed COX-2, while the expression of 15-PGDH was dramatically reduced along the whole extent of the colons derived from the WT, Ras and Ras+5ASA groups. These results suggest that both up-regulation of COX-2 and reduction of 15-PGDH play a role in tumor growth in our murine model, similar to human colon tumor. In addition, we analyzed the non-tumorous colonic area from Ras and Ras+5ASA mice by immunohistochmistry and by western blotting. We also observed down-regulation of COX-2 and up-regulation of 15-PGDH at the protein level in the Ras+5-ASA group. This finding suggests that 5-ASA can exert a preventive effect by reducing COX-2 expression and by inducing 15-PGDH expression synergistically *in vivo*. Since both COX-2 and 15-PGDH are involved in the regulation of PGE2, which is an important growth factor in colorectal cancer, we speculated that 5-ASA would still inhibit tumor growth even if mutations occurred in tumor-suppressor genes or oncogenes resulting in tumor formation. This is very significant in the clinical setting because recent technological breakthroughs allow us to endoscopically cure not only adenomas but even early-stage colorectal cancer.

In this study, we were unable to determine which of the two mechanisms, COX-2 suppression or 15-PGDH induction, made the most significant contribution to the prevention of tumor formation. However, since the WT group mice with DMH treatment, which developed tumors at a rate of 20%, expressed remarkably less 15-PGDH in total colon than the WT mice without the treatment, this suggests that 15-PGDH is the predominant factor, and we propose its regulation as a target for future chemopreventive therapy. Our study is the first report to establish a relationship between the DMH injection and the reduction of 15-PGDH expression.

From a clinical point of view, a previous large multi-center, placebo-controlled trial reported that 1 g/day 5-ASA showed a nonsiginificant trend toward reduction in recurrence of adenomas in subjects with at least three adenomas (36), and another smaller study observed that there was no reduction in size or number of polyps following treatment with 3 g/day balsalazide, a pro-drug of 5-ASA, over a six-month period (37). Since these studies used a low dose of drug and the duration of observation was short, further studies are necessary to properly assess the chemopreventive effect of 5-ASA on sporadic adenomas.

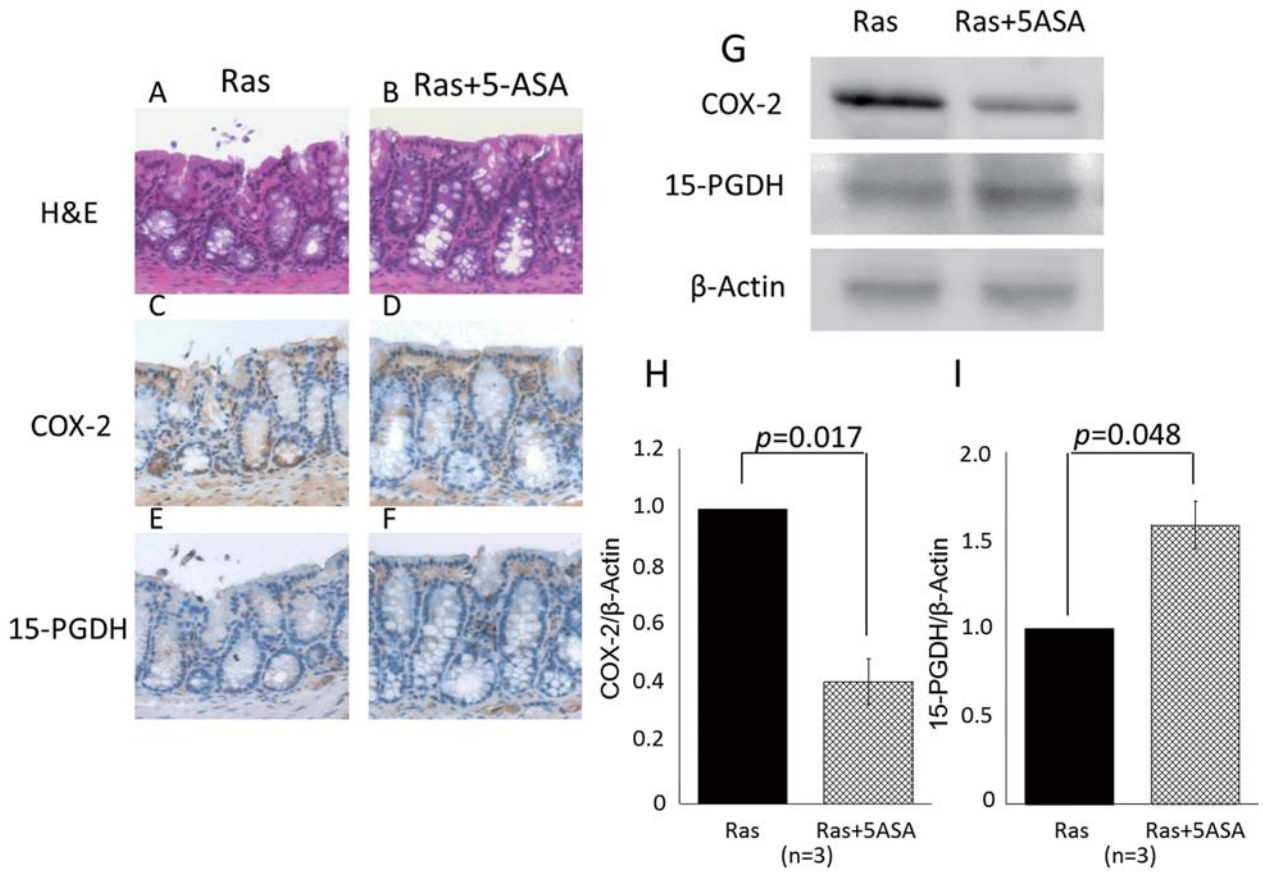

Figure 6. *The effect of 5-aminosalicylic acid (5-ASA) on cyclooxygenase-2 (COX-2) and 15-hydroxyprostaglandin dehydrogenase (15-PGDH) expression in murine colon. Non-tumorous area sections were obtained from the Ras and Ras+5-ASA mice. There was no histopathological difference between these groups by H&E staining (A and B). Specimens were stained for COX-2 and 15-PGDH. COX-2 seemed to be expressed more strongly in the Ras group than in the Ras+5-ASA mice (C and D), while an inverse effect was observed for 15-PGDH (E and F). Representative sections are shown at ×200 magnification. Total protein was extracted from non-tumorous area of colons, and western blotting and quantitative analysis of COX-2 and 15-PGDH expressions, which were normalized to that of beta-actin protein, were performed. 5-ASA significantly reduced the COX-2 expression (p=0.017) and increased the 15-PGDH expression (p=0.048) (G and H). Representative western blots are shown. Values are expressed as a ratio to the average values of the Ras group and are the mean±S.E.M. of three separate experiments.*

In conclusion, we showed that 5-ASA has the potential to suppress COX-2 and induce 15-PGDH protein expression, to exert a chemopreventive effect in a murine colorectal cancer model, using c-Ha-ras transgenic mice, which highly mimics human sporadic colorectal tumor development. Moreover, the dose of 5-ASA used in the *in vitro* and *in vivo* experiments was carefully chosen to reflect efficacy and safety for use in humans. We believe that our results serve as a basis for consideration of a clinical trial to investigate the chemopreventive effect of 5-ASA.

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