Original Article

Sodium salicylate and 5-aminosalicylic acid synergistically inhibit the growth of human colon cancer cells and mouse intestinal polyp-derived cells

Hideki Takakura,[1,2,3] Mano Horinaka,[1,2,*] Ayaka Imai,[1,2] Yuichi Aono,[1,4] Toshimasa Nakao,[1,2] Shingo Miyamoto,[5] Yosuke Iizumi,[1] Motoki Watanabe,[1] Takumi Narita,[1] Hideki Ishikawa,[1] Michihiro Mutoh,[1,5] and Toshiyuki Sakai[1,2]

[1]Department of Molecular-Targeting Prevention and [2]Department of Drug Discovery Medicine, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan
[3]Laboratory of Biopharmaceutics and Pharmacokinetics, Faculty of Pharmaceutical Sciences, Hiroshima International University, 5-1-1 Hirokoshinkai, Kure, Hiroshima 737-0112, Japan
[4]Department of Biomedical Chemistry, Kwansei Gakuin University, Sanda, Hyogo 669-1337, Japan
[5]Epidemiology and Prevention Division, Center for Public Health Sciences, National Cancer Center, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

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As colon cancer is one of the most common cancers in the world, practical prevention strategies for colon cancer are needed. Recently, treatment with aspirin and/or 5-aminosalicylic acid-related agents was reported to reduce the number of intestinal polyps in patients with familial adenomatous polyposis. To evaluate the mechanism of aspirin and 5-aminosalicylic acid for suppressing the colon polyp growth, single and combined effects of 5-aminosalicylic acid and sodium salicylate (metabolite of aspirin) were tested in the two human colon cancer cells with different cyclooxygenase-2 expression levels and intestinal polyp-derived cells from familial adenomatous polyposis model mouse. The combination induced cell-cycle arrest at the G1 phase along with inhibition of cell growth and colony-forming ability in these cells. The combination reduced cyclin D1 via proteasomal degradation and activated retinoblastoma protein. The combination inhibited the colony-forming ability of mouse colonic mucosa cells by about 50% and the colony-forming ability of mouse intestinal polyp-derived cells by about 90%. The expression level of cyclin D1 in colon mucosa cells was lower than that in intestinal polyp-derived cells. These results suggest that this combination may be more effective in inhibiting cell growth of intestinal polyps through cyclin D1 down-regulation.

***Key Words*: aspirin, sodium salicylate, mesalazine, cyclin D1, colon cancer**

Colon cancer (CRC) is one of the most common cancers in the world. The number of CRC patients worldwide was 1.8 million in 2018, the third highest number of patients in malignant neoplasms.[1] Therefore, a practical prevention strategy for CRC is needed to extend healthy life expectancy.

In previous studies, aspirin (acetylsalicylic acid, ASA) was shown to be one of the most promising drugs for CRC prevention, and various clinical trials reported that ASA reduced the risk of CRC.[2–4] ASA was developed in 1897 as a prodrug to reduce side effects of salicylic acid.[5] Currently, salicylic acid has been replaced by ASA as an antipyretic analgesic in the medical setting. ASA is metabolized to salicylic acid by esterase in the intestinal epithelium and liver.[6,7] Several studies[8,9] showed that salicylic acid has inhibitory effects on cell growth in human CRC cell lines. Several studies[9,10] suggest that salicylic acid has potential as an active metabolite for CRC prevention.

In addition to ASA, several drugs are reportedly expected to prevent CRC.[11–14] Among them, we recently reported that mesalazine (5-aminosalicylic acid, 5-ASA), which is known to be a lipoxygenase inhibitor and weak cyclooxygenase (COX) inhibitor,[15] tended to reduce the number of colon polyps in familial adenomatous polyposis (FAP) patients with ulcerative colitis (UC) in a clinical trial.[14] 5-ASA-related agents, such as salazosulfapyridine, Pentasa® and Lialda®, are standard drugs for the treatment of UC. UC is known to be a high-risk disease for CRC. It is important to realize that UC is at high risk for CRC in order to prevent CRC.[16] Moreover, 5-ASA has been reported to inhibit cell growth depending on the expression level of COX-2 protein in human CRC cells.[17] To investigate whether the effect of this combination therapy depends on the expression level of COX-2, we selected colon cancer cell lines with different COX-2 expression levels. HT-29 cells were used as COX-2 expressing cells and HCT-15 cells were used as COX-2 deficient cells in this study. In addition, case-control studies have shown that 5-ASA administration significantly reduced the relative risk of CRC in UC patients.[18–20] Therefore, 5-ASA has attracted a lot of attention as a candidate drug for CRC prevention.

On the other hand, patients with FAP are known to be at increased risk of CRC due to mass development of precancerous lesions.[21] The incidence of CRC is 50% and almost 100% in patients aged in their 40s and 70s, respectively.[22] Cyclin D1 protein is more highly expressed in CRC lesions in patients with CRC[23] and polyps in patients with FAP[24] than in each normal intestinal epithelium, and has important functions in cell cycle progression. A high level of cyclin D1, associated with mutations in the adenomatous polyposis coli gene (*APC*), inactivates retinoblastoma (RB) protein and promotes the G1-S phase transition of the cell cycle.[25–28]

We recently performed a randomized, double-blind, placebo-controlled multi-center trial with a 2 × 2 factorial design to determine the individual and joint effects of low-dose ASA and mesalazine on the recurrence of colon polyps in Japanese FAP patients without a history of colectomy.[29] This trial is the first randomized trial that showed low-dose ASA suppresses the formation of colon polyps in FAP patients, as far as we know,

*To whom correspondence should be addressed.
E-mail: m-hori@koto.kpu-m.ac.jp

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and this is the largest trial of FAP patients over age 21 without a history of colectomy. In this trial, mesalazine only tended to reduce the recurrence of colon polyps. Clinical trial could not be able to reveal the mechanism of tumor suppressive effects of ASA and/or mesalazine. Thus, this study is aimed to evaluate the effects and mechanism of sodium solicylate (SS) and 5-ASA on intestinal polyp-derived cells from mouse and human CRC cells.

Materials and Methods

Cell culture. Two human colon cancer cell lines, HT-29 and HCT-15 cells, were purchased from the U.S. National Cancer Institute. Three cells, small intestinal polyp-derived cells (SIP), colonic polyp-derived cells (CP), and colon mucosa (CM) cells, were derived from intestinal polyps and mucosa of 40-week-old female C57BL/6-*APC*$^{Min/+}$ mouse. Briefly, the removed intestinal polyps were excised into small pieces and normal appearing mucosa were smeared, and then, both were placed onto collagen coated plates. After 4 weeks of incubation, cells were trypsinized and passed into normal tissue culture plates. All experiments were carried out according to the "Guidelines for Animal Experiments in the National Cancer Center". Animal studies were approved by the Institutional Ethics Review Committee for Animal Experimentation of the National Cancer Center (T17-017-M03). All cells were cultured at 37°C in an atmosphere containing 5% CO_2.

Cell culture medium. HT-29 and HCT-15 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Nissui, Tokyo, Japan). Culture medium was supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. SIP, CP, and CM cells were cultured in the maintaining medium as shown below. The maintaining medium contains penicillin/streptomycin, HEPES, Glutamax, N2 Supplement, B27 Supplement (Life Technologies, Carlsbad, CA), *N*-acetylcysteine, gastrin, nicotinamide, SB202190 (Sigma-Aldrich, St. Louis, MO), EGF, dorsomorphin, Y-27632, A-83-01 (Wako Chemicals, Osaka, Japan), GSK-3 inhibitor XV (Santa Cruz Biotechnology, Dallas, TX), CHIR99021 (Axon Medchem, Groningen, Netherlands), and 2% FBS in advanced DMEM/F12 (Life Technologies). This medium is based on previous reports.[30,31]

Reagents. SS (#S2679; Sigma-Aldrich) and 5-aminosalicylic acid (5-ASA, #A3537; Sigma-Aldrich) were dissolved in optimal medium for each cell line. Acetylsalicylic acid (ASA, #A5376; Sigma-Aldrich), zVAD-fmk (#FMK001; R&D Systems, Minneapolis, MN), and MG132 (#3175-V, Peptide Institute, Osaka, Japan) were dissolved in dimethyl sulfoxide (DMSO).

Cell growth assay. HT-29 and HCT-15 cells were plated at a density of 3×10^3 cells per well, and SIP and CP cells were plated at a density of 1×10^3 cells per well in 96-well plates. After incubation for 24 h, the cells were treated with SS, 5-ASA, and ASA for 72 h. After washing with PBS, the cells were incubated with Cell Counting Kit-F (CCK-F) reagent (Dojindo, Kumamoto, Japan) for 30 min at 37°C and fluorescence intensity was measured using a SpectraMax M2e (Molecular Devices, San Jose, CA).

Analysis of cell cycle and detection of apoptosis. HT-29 and HCT-15 cells were seeded at a density of 4×10^4 cells per well, and CP cells were plated at a density of 1×10^4 cells per well in 6-well plates. After incubation for 24 h, cells were treated with SS and 5-ASA for 24 or 72 h. The cells were harvested by trypsinization. After centrifugation with PBS, the cells were resuspended in PBS containing 0.1% Triton X-100 and 25 µg/ml propidium iodide (Sigma-Aldrich). DNA content was measured using a FACSCalibur or BD Accuri C6 Plus flow cytometer (Becton Dickinson, Franklin Lakes , NW). For each experiment, 10,000 cells were analyzed. Cell Quest software (Becton Dickinson) and ModFit LT ver. 2.0 software package (Verity Software House, Topsham, ME) were used to analyze the data. DNA fragmentation was quantified by the percentage of hypodiploid DNA (sub-G1).

Protein and RNA preparation. HT-29, HCT-15, SIP, CP, and CM cells were seeded at a density of 2×10^5 cells per dish in 100-mm dishes. After incubation for 24 or 48 h, cells were treated with SS and 5-ASA for 24 h. For protein preparation, the cells were solubilized in lysis buffer (50 mM Tris-HCl, 1% SDS, 2 µg/ml leupeptin, 2 µg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). Total RNA was extracted from the cells using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan) and RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA).

Western blot. Cell extracts were subjected to SDS-PAGE under reducing conditions, and then proteins were transferred to polyvinylidene fluoride transfer membranes. The membranes were incubated with 3% BSA in TBST (0.05% Tween 20 in TBS) for 1 h at room temperature. Then, membranes were incubated with anti-human phospho-RB (Ser780) (#9307; Cell Signaling Technology, Danvers, MA) and anti-cyclin D1 (#06-137; Upstate Biotechnology, Lake Placid, NY) antibodies. The membranes were washed and incubated with HRP-conjugated anti-rabbit IgG antibody (#NA934V) or HRP-conjugated anti-mouse IgG antibody (#NA931V) (GE Healthcare, Piscataway, NJ). The blots were visualized by enhanced chemiluminescence and detected using a ChemiDoc system (Bio-Rad, Hercules, CA). Each membrane was re-probed with anti-β-actin antibody (#A5441; Sigma-Aldrich) to confirm equal loading of the proteins.

Quantitative reverse transcription-PCR (qRT-PCR). Total RNA was reverse transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and oligo (dT) primers (Toyobo, Osaka, Japan) according to the manufacturer's instructions. qRT-PCR was performed with QuantStudio 3 Real-Time PCR System (Thermo Scientific) to measure the expression levels of cyclin D1 and β2MG. Primers and TaqMan probes for cyclin D1 (#Hs00765553_m1) and β2MG (#Hs00984230_m1) were used with the TaqMan Gene Expression Assay. qRT-PCR results were expressed as cyclin D1/β2MG as an internal standard concentration ratio.

Gene expression omnibus data analysis (microarray analysis). Micro array dataset of CRC and adjacent normal intestinal epithelium from 17 patients with CRC were obtained in previous report.[32] All samples were resected by surgical treatment for CRC. We obtained the gene expression data sets which are submitted to Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/, accession number GSE32323). Differential gene expression analysis was performed by GEO2R.

Colony formation assay (protocol for seeding before treatment). HT-29, HCT-15, SIP, and CP cells were seeded in 6-well plates at a density of 4×10^2 cells per well, and CM cells were seeded in 6-well plates at a density of 1×10^4 cells per well. Twenty-four h after seeding, HT-29, HCT-15, SIP, and CP cells were treated with SS and 5-ASA for 72 h. Forty-eight h after seeding, CM cells were treated with SS and 5-ASA for 72 h. After replacing the medium with fresh medium, the HT-29, HCT-15, SIP, and CP cells were cultured for 3 days, CM cells were cultured for 4 days. The colonies were then fixed with 10% formalin and stained with crystal violet. All assays were performed in triplicate. These colony areas in each well were quantified using a SpectraMax iD3 microplate reader (Molecular Devices).

Colony formation assay (protocol for seeding after treatment). For CP cells, another protocol was used: CP cells were seeded in 6-well plates at a density of 1×10^4 cells per well. After a 24-h incubation, cells were treated with SS and 5-ASA for 24 h. The cells were then reseeded in 6-well plates at a

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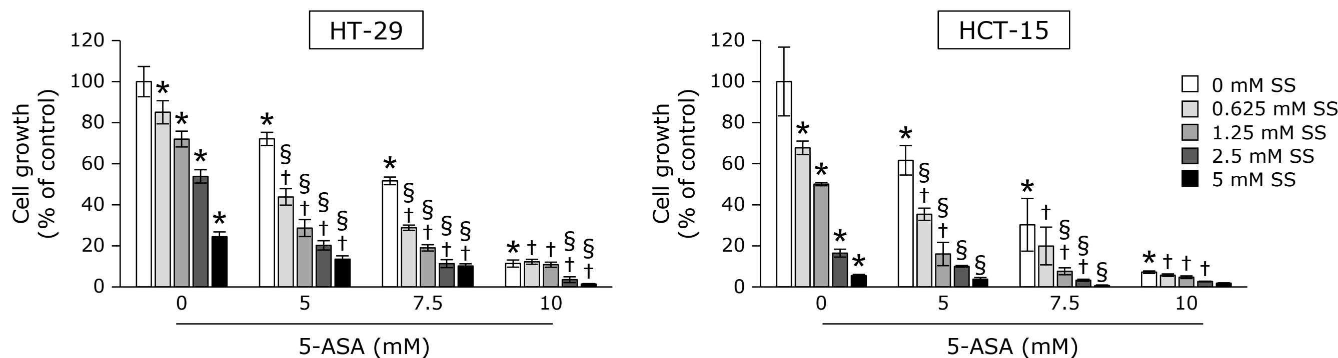

Fig. 1. Inhibitory effects of cell growth in human colon cancer cells by SS and 5-ASA. HT-29 and HCT-15 cells were treated with various concentrations of SS and/or 5-ASA for 72 h. Cell growth was measured by CCK-F. Data are means ± SEM; $n = 3$; *$p<0.05$, relative to non-treated control; †$p<0.05$, relative to corresponding SS-treated control; §$p<0.05$, relative to corresponding 5-ASA-treated control. *P* values were calculated by Tukey's multiple comparisons test.

Table 1. The combination index (CI) of SS and 5-ASA was calculated in HT-29 and HCT-15 cells

CI (HT-29)	0.625 mM SS	1.25 mM SS	2.5 mM SS	5 mM SS
5 mM 5-ASA	0.924	0.869	0.917	1.01
CI (HCT-15)	0.625 mM SS	1.25 mM SS	2.5 mM SS	5 mM SS
5 mM 5-ASA	1.18	1.062	1.219	1.183

density of 4×10^2 cells per well. The colonies were cultured 7 days after reseeding. The colonies were then fixed with 10% formalin and stained with crystal violet. All assays were performed in triplicate. These colony areas in each well were quantified using a SpectraMax iD3 microplate reader (Molecular Devices).

ELISA. CP cells were plated at a density of 1×10^3 cells per well in 96-well plates. After incubation for 24 h, the cells were treated with SS, 5-ASA, and ASA for 24 h. Cell culture supernatants were collected, and the expression of tumor necrosis factor α (TNF-α) was measured by ELISA using the mouse TNF-α ELISA kit (R&D Systems) according to the manufacturer's instructions.

Combination index. Combination index (CI) was calculated using CalcuSyn software program (Biosoft, Ferguson, MO) according to the manufacturer's protocol. Synergistic inhibitory effect (with CI <1.0) was defined as more than the expected additive inhibitory effect.

Statistical analysis. All data are presented as mean ± SEM. Differences among groups were evaluated by one-way or two-way analysis of variance followed by Tukey's multiple comparisons test, Sidak's multiple comparisons test, or Student's *t* test. *P* values <0.05 were considered significant. Computations were performed with GraphPad Prism ver. 8.3.1 (GraphPad Software, San Diego, CA).

Results

Combined treatment with SS and 5-ASA inhibits cell growth of human colon cancer cells. To confirm the combined effect of SS and 5-ASA, we firstly analyzed the growth inhibitory effects of these agents alone. SS or 5-ASA inhibited the cell growth of human colon cancer HT-29 and HCT-15 cells in a dose-dependent manner (Supplemental Fig. 1*). Next, we evaluated the inhibitory effect of co-treatment with SS and 5-ASA at various concentrations on cell growth in the cells. The combination of SS and 5-ASA significantly reduced the cell growth of HT-29 and HCT-15 cells compared to multiple concentrations of either treatment alone (Fig. 1 and Table 1). Subsequent verification was evaluated by this combination of ratios which show significant difference in the inhibitory effect of each single agent. Since ASA, but not SS, is clinically used, we additionally evaluated the combined effects of SS or ASA at various concentrations with 5 mM 5-ASA in the cells. As shown in Supplemental Fig. 2*, both the combination of ASA & 5-ASA and SS & 5-ASA were effective in inhibiting the cell growth of HT-29 and HCT-15 cells.

Combined treatment with SS and 5-ASA induces cell cycle arrest with the suppression of cyclin D1 in human colon cancer cells. To clarify the mechanisms of the combined effects of SS and 5-ASA on cell growth, we analyzed the cell cycle by flow cytometry. We firstly analyzed the effects on the cell cycle by these agents alone. SS or 5-ASA induced cell-cycle arrest at the G1 phase in HT-29 and HCT-15 cells in a dose-dependent manner (Supplemental Fig. 3*). The combination further induced cell-cycle arrest at the G1 phase in HT-29 and HCT-15 cells after 24 h (Fig. 2A) and 72 h (Fig. 2B) compared to each single treatment. Furthermore, combination treatment induced caspase-dependent apoptosis in only HT-29 cells after 72 h (Supplemental Fig. 4*).

We next investigated the expression level of G1 arrest-related proteins by western blot analysis. Combination treatment converted the phosphorylation status of RB protein at serine 780 to the hypo-phosphorylated active state and reduced cyclin D1 expression in both cells (Fig. 2C).

Combined treatment with SS and 5-ASA reduces expression level of cyclin D1 via proteasomal degradation. To investigate how this combination decreased the cyclin D1 protein in human colon cancer cells, cyclin D1 mRNA expression level in these cell lines were assessed by qRT-PCR. As shown in Fig. 3A, either SS or 5-ASA caused an approximately 30% decrease in cyclin D1 expression compared to the control, and the combination resulted in an approximately 60% decrease in cyclin D1 expression in HT-29 cells. However, cyclin D1 mRNA expres-

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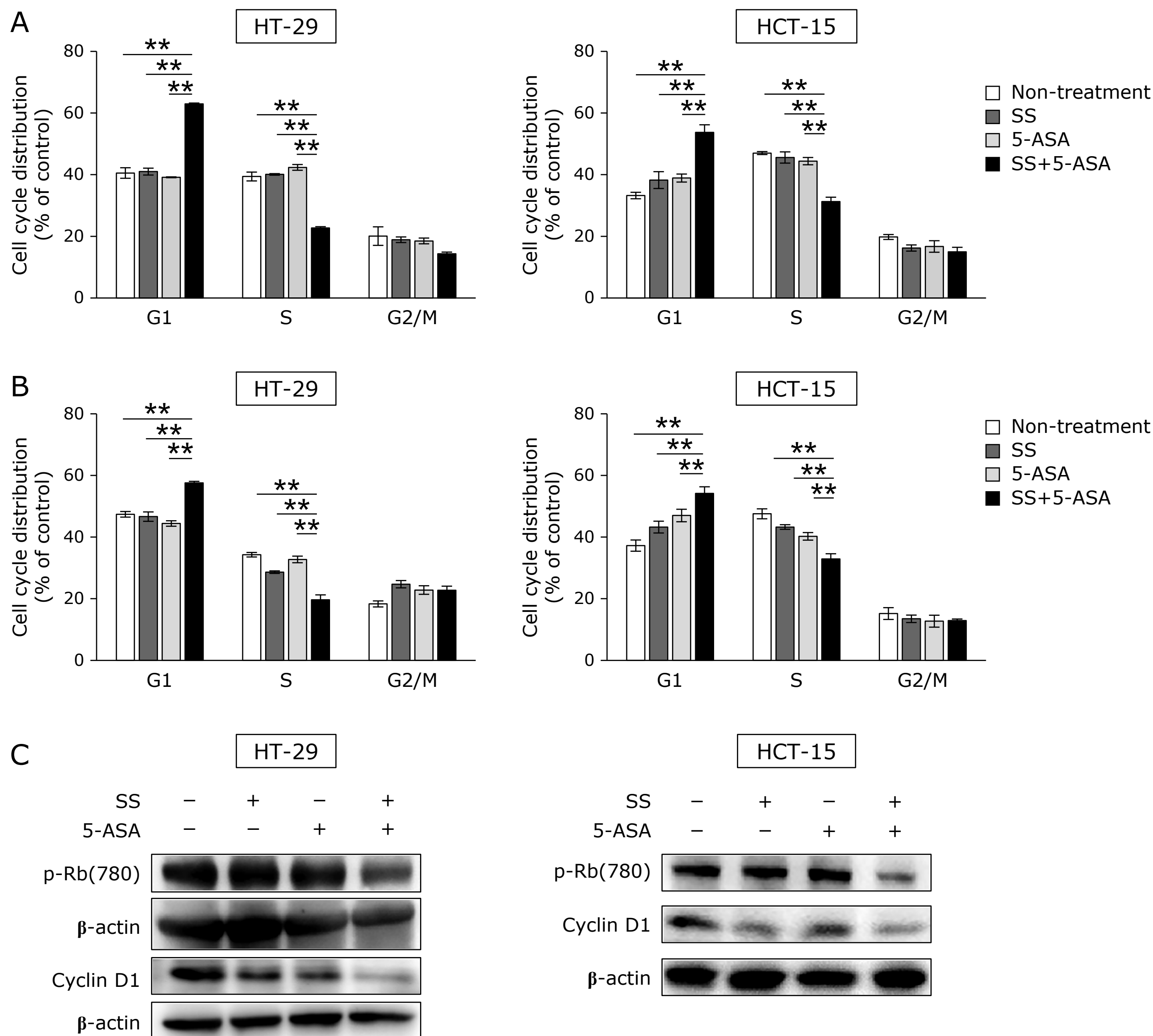

Fig. 2. Effects of combined treatment on cell cycle and expression of G1 arrest-related proteins in human colon cancer cells. HT-29 and HCT-15 cells were treated with 1.25 mM SS and/or 5 mM 5-ASA or alone. (A) and (B), Cell cycle analysis of these cells by flow cytometry after 24 h (A) or 72 h (B). Data are means ± SEM; $n = 3$; ** $p<0.01$. (C) Protein was harvested and prepared from these cells after 24 h. Protein expression levels were examined by western blot analysis. β-actin was used for the loading control. *P* values were calculated by Tukey's multiple comparisons test (A, B).

sion was not changed by the single and combined treatments in HCT-15 cells. Thus, we next aimed to investigate whether the down-regulated cyclin D1 could be explained by proteasomal degradation. To this aim, the cells were treated with the proteasome inhibitor MG132. The results showed that MG132 canceled the combination-induced reductions of cyclin D1 expression in both HT-29 and HCT-15 cells (Fig. 3B).

Combined treatment with SS and 5-ASA reduces colony-forming ability of human colon cancer cells. To assess the effect of the combined treatment with SS and 5-ASA on colony-forming ability, we performed colony formation assays of the HT-29 and HCT-15 cells. The combined treatment clearly reduced colony-forming ability in both human colon cancer cell lines (Fig. 4A). In addition to these visual analyses, we quantified these colony areas and performed statistical analyses, and found that the combined treatment significantly reduced colony area compared to each single treatment (Fig. 4B).

Combined treatment with SS and 5-ASA additively/synergistically inhibits cell growth in mouse intestinal polyp-derived cells. Since there are no commercially available cells derived from FAP patients, we used the cells derived from the *APC*$^{Min/+}$ mouse, a model mouse of FAP.[33] We established intestinal polyp-derived cells (SIP and CP cells) from *APC*$^{Min/+}$ mouse. Firstly, we assessed the inhibitory effects on cell growth by these agents alone. SS or 5-ASA inhibited the cell growth of mouse intestinal polyp-derived cells in a dose-dependent manner (Fig. 5A). The 5-ASA dose for significant inhibitory effect on cell growth was higher in mouse intestinal polyp-derived cells than in human colon cancer cells (Fig. 5A). Next, we examined the inhibitory effect of co-treatment with SS and 5-ASA at various concentrations on cell growth in those mouse intestinal polyp-derived cells. As a result, the combined treatment signifi-

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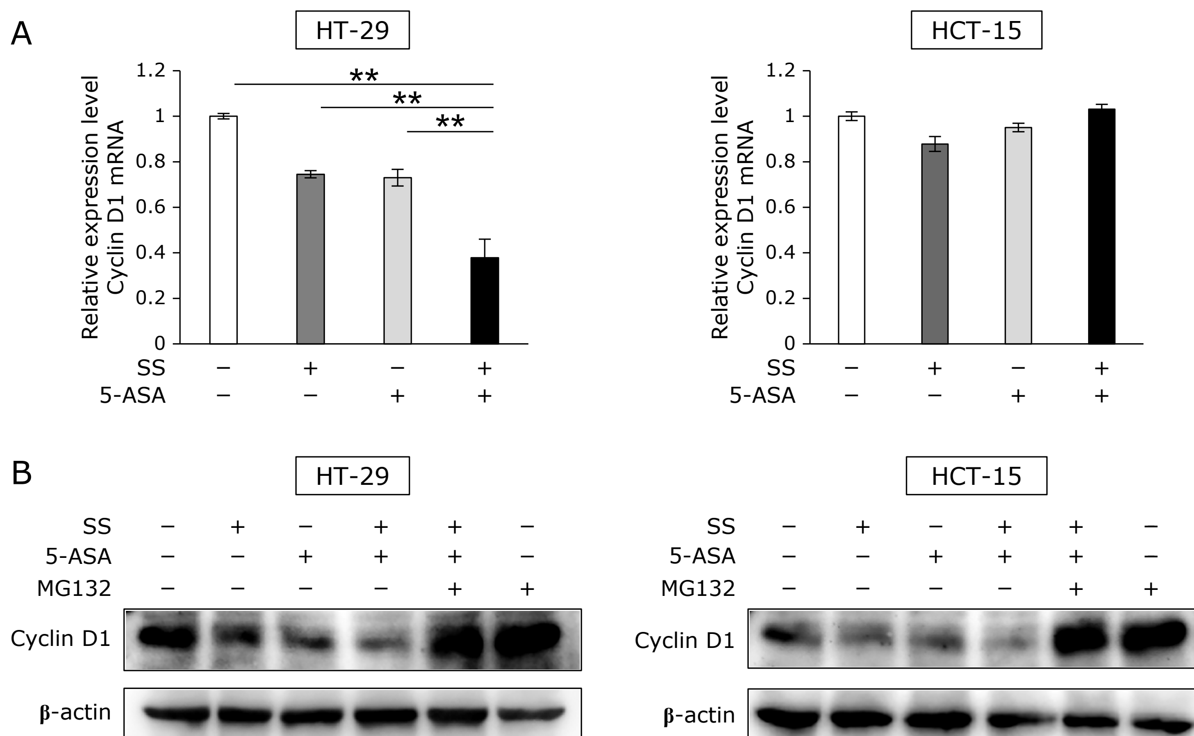

Fig. 3. Analysis of combined treatment effects on the reduction of cyclin D1 expression in human colon cancer cells. (A) HT-29 and HCT-15 cells were treated with 1.25 mM SS and/or 5 mM 5-ASA for 24 h. The cyclin D1 mRNA expression levels in these cells were normalized using β2MG. Data are means ± SEM; $n = 3$; $^{**}p<0.01$. (B) HT-29 and HCT-15 cells were treated with 1.25 mM SS and/or 5 mM 5-ASA, with or without 20 μM MG132 for 24 h. The expression level of cyclin D1 protein in these cells were identified by Western blot analysis. β-Actin was used for the loading control. *P* values were calculated by Tukey's multiple comparisons test (A).

cantly reduced the cell growth of both SIP and CP cells than any of the treatments alone at various concentrations (Fig. 5B). Moreover, the combined treatment indicated that additive/synergistic inhibitory effects on both cells at several ratios of SS and 5-ASA (Table 2). In addition, we evaluated the combined effects of 10 mM 5-ASA plus SS or ASA at various concentrations in CP cells. As a result, the combination of ASA and 5-ASA, as well as that of SS and 5-ASA, inhibited cell growth of CP cells (Supplemental Fig. 5*).

Next, we evaluated the combined effects of SS and 5-ASA on TNF-α expression levels in culture medium of CP cells by ELISA. As a result, the combination reduced the production of TNF-α in CP cells after 24 h (Fig. 6).

Combined treatment with SS and 5-ASA reduces colony-forming ability of mouse intestinal polyp-derived cells. The combined treatment for 24 h induced cell-cycle arrest at the G1 phase in CP cells in both mouse intestinal polyp and human colon cancer cells (Supplemental Fig. 6*). Next, we evaluated the effects of the combined treatment with SS and 5-ASA on colony-forming ability of CM, SIP, and CP cells. As a result, the combined treatment significantly reduced colony area of CM, SIP, and CP cells (Fig. 7A and B). In addition, we also evaluated the treatment on colony-forming ability of CP cells by another protocol in which cells were reseeded after the treatment with SS and 5-ASA. As shown in Supplemental Fig. 7*, the combinatory treatment tended to inhibit colony formation compared to that of each single treatment. The difference between the protocols in Fig. 7 and Supplemental Fig. 7* is the timing of drug treatment and plating. In Supplemental Fig. 7*, the drug treatment is performed before plating because the purpose is to verify the effect of the drug on cell adhesion ability. CP cells were established from intestinal polyps taken from C57BL/6-*APC*$^{Min/+}$ mouse, a well-known research model for FAP and are an important test subject in this study. Many studies have shown that polyps play an important role in the development of CRC. The purpose of this study is to obtain basic research data for CRC chemoprevention research. Therefore, our aim was to collect experimental data using CP cells among the cell lines used in this study. However, the inhibition rate of colony area of CM was clearly lower compared to that of SIP and CP cells (Fig. 7A and B). Furthermore, the expression level of cyclin D1 in CM cells was lower than that in SIP and CP cells (Fig. 7C).

Discussion

In this study, we aimed to answer our clinical question why ASA and/or mesalazine suppressed the development of colon tumor in FAP patients without a history of colectomy. The mechanism of tumor suppressive effects could be explained by the suppression of cyclin D1.

5-ASA has an inhibitory effect on the production of inflammatory cytokines and is known as an anti-inflammatory drug used for inflammatory bowel diseases such as UC. Dextran sulfate sodium (DSS)-exposed mice have been widely used in experimental models of UC.[34] A previous report showed that 5-ASA suppressed the up-regulation of COX-2 mRNA expression level in DSS-induced colitis.[35] 5-ASA has been reported to inhibit cell growth depending on the expression level of COX-2 protein in human CRC cells.[17] Based on these previous studies, we speculated that the inhibitory effects of 5-ASA-related agents on cell growth depend on the inflammatory state in CRC cells. However, 5-ASA showed COX-2-independent inhibitory effects on cell growth of HT-29 cells expressing COX-2 as well as COX-2-deficient HCT-15 cells[36] (Supplemental Fig. 1*).

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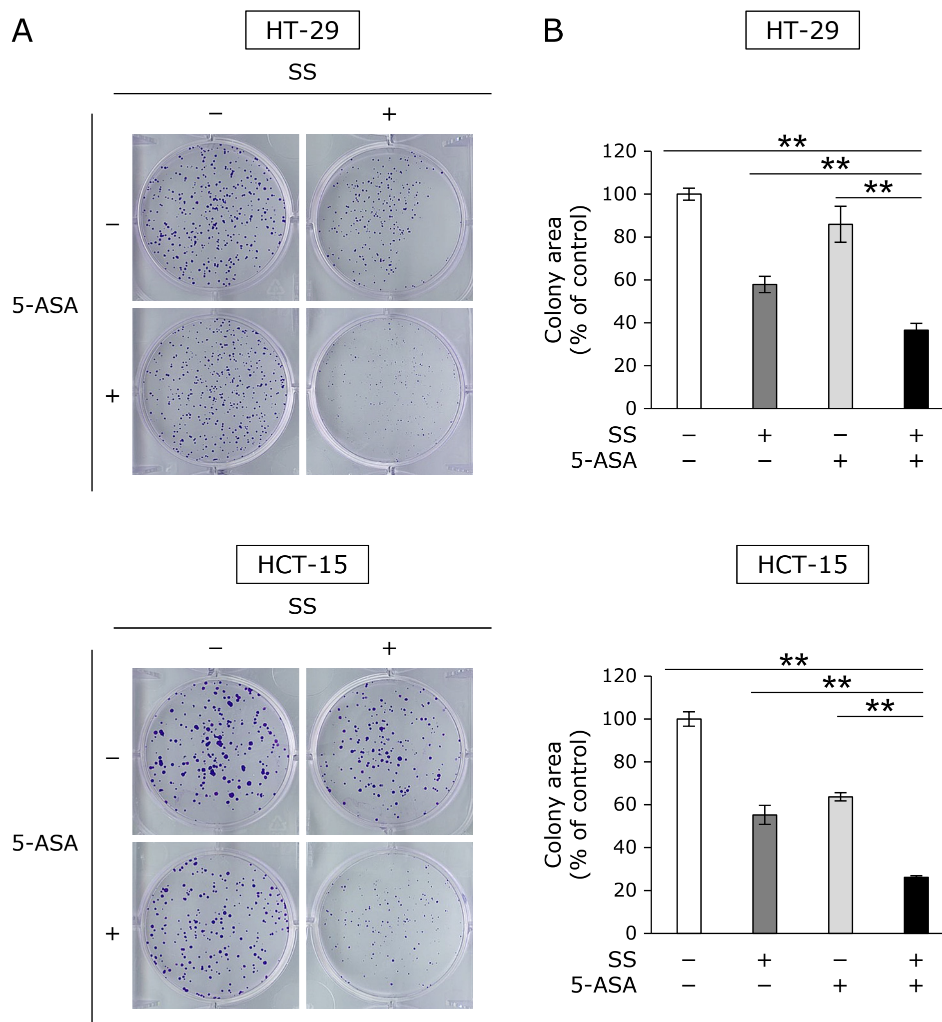

Fig. 4. Effects of the combined treatment on colony-forming ability in human colon cancer cells. HT-29 and HCT-15 cells were treated with 1.25 mM SS and/or 5 mM 5-ASA for 72 h and incubated for 4 days. The colonies were stained by crystal violet. (A) Representative images of stained colonies are shown. (B) Colony area was quantified using SpectraMax iD3. Data are means ± SEM; *n* = 3; ***p*<0.01. *P* values were calculated by Tukey's multiple comparisons test (B).

A previous study reported that SS and ASA inhibited cell growth induced G1-phase arrest in human CRC cells *in vitro*.[8] Our results suggest that salicylic acid is an active metabolite of ASA for anti-cancer effects and plays an important role in combined treatment with 5-ASA (Fig. 1 and Supplemental Fig. 2*). In the present study, SS, which is thought to be an active metabolite of ASA, induced G1-phase arrest in HT-29 and HCT-15 cells (Supplemental Fig. 3*). Several researchers showed that 5-ASA induced S-phase arrest in HT-29 cells,[37,38] but in our experimental conditions, no such induction was observed in HT-29 and HCT-15 cells (Supplemental Fig. 3*). We previously reported that several promising natural products and drugs for prevention of CRC reduced expression level of cyclin D1 and induced G1-phase arrest in human CRC cells.[39–41] We speculate that cell-cycle arrest at the G1 phase via reducing expression level of cyclin D1 was an important event for the combined effects of SS and 5-ASA in suppressing CRC development (Fig. 2).

Cyclin D1 mRNA expression level is higher in the CRC region than in normal intestinal epithelium.[42] Expression level of cyclin D1 mRNA in cancer regions were higher than that in normal intestinal epithelium in CRC patients (Supplemental Fig. 8*). *APC* gene is known to be an important factor controlling cyclin D1 mRNA expression level via Wnt/β-catenin signaling.[28] HT-29 and HCT-15 cells are considered as *APC*-mutated colon cancer cell lines.[43] The combination suppressed cyclin D1 mRNA levels in HT-29 cells but not in HCT-15 cells (Fig. 3A). This mechanism may not be common in CRC cells.

Accelerated degradation of cyclin D1 protein could be one of the promising strategies for CRC prevention. Previously, researchers reported that cyclin D1 protein is unstable and is degraded ubiquitin-dependently via proteasomes in human cells.[44] Promising natural products and drugs for prevention of CRC, such as ASA, resveratrol, and troglitazone, has been reported to induce cyclin D1 degradation in human cancer cell lines.[44] We speculate that proteasome degradation is one of important mechanisms for suppressing the expression of cyclin D1 protein by the combined treatment (Fig. 3B).

Cyclin D1 is important for development and progression of colon adenomas. Cyclin D1 binds to cyclin-dependent kinase (CDK) 4 or 6 and advances from the G1 phase to the S phase of the cell cycle. Cyclin D1-CDK4 or cyclin D1-CDK6 complex promotes phosphorylation and inactivation of RB.[25–27] Cyclin D1 mRNA and protein expression levels in polyp regions are

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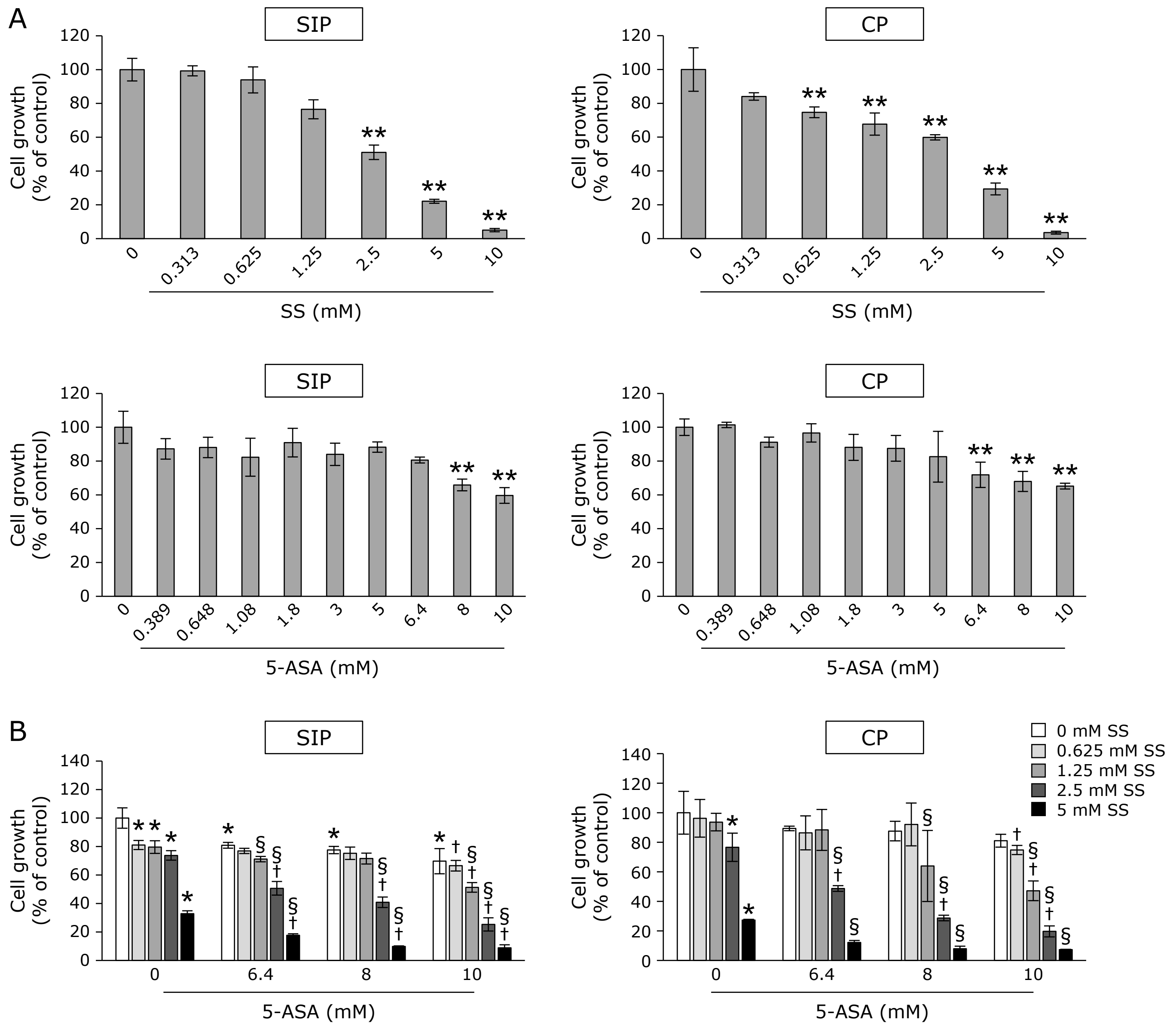

Fig. 5. The combined treatment with SS and 5-ASA additively/synergistically inhibits the cell growth in mouse intestinal polyp-derived cells. (A) SIP and CP cells were treated with various concentrations of SS or 5-ASA for 72 h. Cell growth was measured by CCK-F. Data are means ± SEM; *n* = 3; ***p*<0.01; relative to non-treated control. (B) SIP and CP cells were treated with various concentrations of SS and/or 5-ASA for 72 h. Cell growth was measured by CCK-F. Data are means ± SEM; *n* = 3; **p*<0.05, relative to non-treated control; †*p*<0.05, relative to corresponding SS-treated control; §*p*<0.05; relative to corresponding 5-ASA-treated control. *P* values were calculated by Tukey's multiple comparisons test (A, B).

Table 2. The combination index (CI) of SS and 5-ASA was calculated in SIP and CP cells

CI (SIP)	0.625 mM SS	1.25 mM SS	2.5 mM SS	5 mM SS
10 mM 5-ASA	1.195	0.885	0.452	0.216
CI (CP)	0.625 mM SS	1.25 mM SS	2.5 mM SS	5 mM SS
10 mM 5-ASA	1.044	0.66	0.483	0.456

higher than those in normal intestinal epithelium.[45] A lack of cyclin D1 gene in *APC*$^{\mathrm{Min/+}}$ mice reduces the number of intestinal polyps.[46] Therefore, cyclin D1 has potential as one of the promising therapeutic targets for intestinal polyps. Based on expression level of cyclin D1 protein, SIP, CP, and CM cells are considered that reproducing the characteristics of polyp or normal intestinal epithelium tissue (Fig. 7C). These results suggest that these cells could be a useful research tool for FAP. Interestingly, the inhibitory effect of the combination treatment was inversely correlated with the level of cyclin D1 expression in

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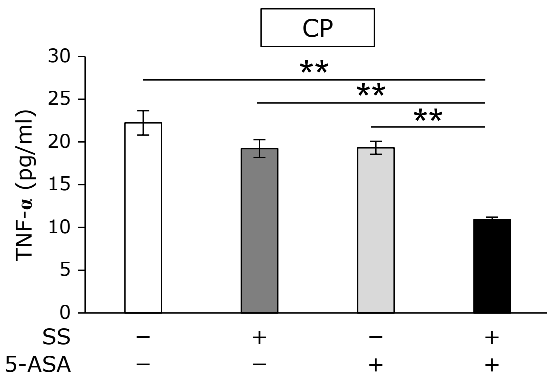

Fig. 6. The combined treatment with SS and 5-ASA inhibits the expression of TNF-α in mouse intestinal polyp-derived cells. CP cells were treated with 2.5 mM SS and/or 10 mM 5-ASA for 24 h. TNF-α concentration in the cell culture supernatants were measured by ELISA. Data are means ± SEM; *n* = 3; $^{**}p<0.01$. *P* values were calculated by Tukey's multiple comparisons test.

mouse intestinal polyp-derived cells (Fig. 7B and C). In this study, the combined treatment with SS and 5-ASA significantly reduced the cell growth of human CRC or mouse intestinal polyp-derived cells compared to either treatment alone (Fig. 1 and 5B). However, the combination showed a clearly synergistic inhibitory effect on mouse intestinal polyp-derived cells compared with CRC cells at several ratios of SS and 5-ASA (Table 1 and 2). Thus, the combination may be more promising as a treatment for intestinal polyps.

TNF-α is a pro-inflammatory cytokine and is known to induce cyclin D1 expression level in CRC cells.[47] Several researchers reported that TNF-α promoted cell cycle transition to the G1-S phase in several cancer cell lines.[48,49] We speculate that down-regulation of TNF-α expression in the culture medium may also contribute to the additive/synergistic inhibitory effects of cell growth via induced cell-cycle arrest at the G1 phase on mouse intestinal polyp-derived cells (Fig. 6 and Supplemental Fig. 6*).

SS was reported to do not change peroxisome proliferator-activated receptor γ (PPAR γ) activity.[50,51] In contrast, 5-ASA was reported to enhance PPAR γ activity and expression.[52] It is reported that PPAR γ have effect of down-regulated cyclin D1 expression level via proteosome degradation.[44] We speculate that SS and 5-ASA showed the combined effect to reduce cyclin D1 expression through the mechanisms above. In conclusion, the

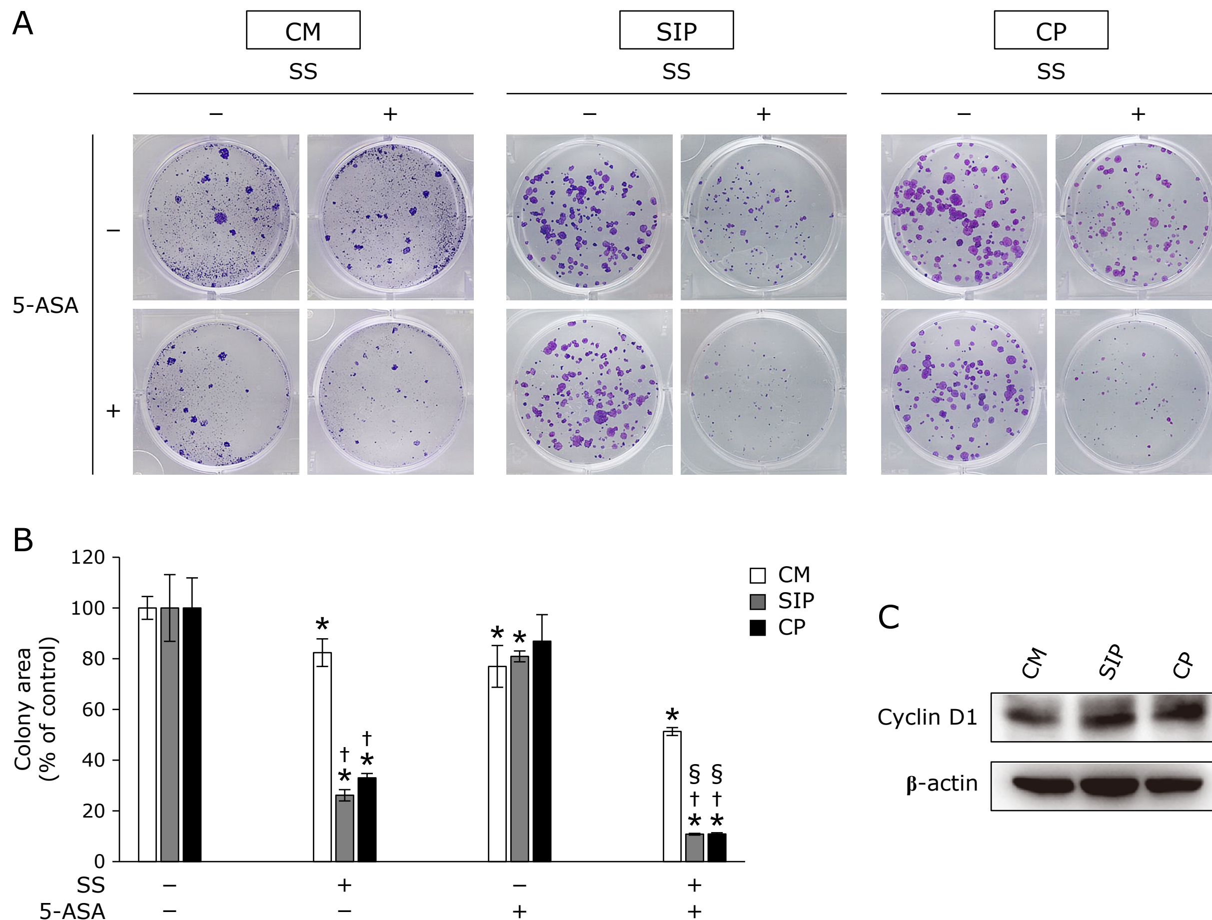

Fig. 7. The combined treatment with SS and 5-ASA reduces colony-forming ability of mouse intestinal polyp-derived cells. CM, SIP, and CP cells were treated with 2.5 mM SS and/or 10 mM 5-ASA for 72 h and incubated for 3 days. The colonies were stained by crystal violet. (A) Representative images of stained colonies are shown. (B) Colony area was quantified using SpectraMax iD3. Data are means ± SEM; *n* = 3; $^{*}p<0.05$, relative to non-treated control; $^{†}p<0.05$, relative to corresponding SS- or SS and 5-ASA-treated CM; $^{§}p<0.05$, relative to corresponding SS-treated control. (C) Cells were seeded and incubated for 24 h (SIP and CP) or 48 h (CM). The cyclin D1 protein expression levels in these cells were identified by western blot analysis. β-Actin was used for the loading control. *P* values were calculated by Tukey's multiple comparisons test and Sidak's multiple comparisons test.

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combination of SS plus 5-ASA treatment suppressed the cell growth of both CRC cells and intestinal polyp-derived cells. This study provided one of the mechanisms of the growth-inhibitory effect of ASA and 5-ASA on CRC cells and intestinal polyp cells. The results raise a possibility that the combination of ASA and 5-ASA may be effective as a preventive method for CRC in FAP patients.

Author Contributions

Conceptualization, HT, MH, HI, MM, and TS; methodology, HT, MH, YA, TNakao, SM, MM, and TS; investigation, HT; resources, SM and MM; data curation, HT, MH, AI, YA, TNakao, YI, MW, MM, and TS; writing–original draft preparation, HT; writing–review and editing, MH, AI, YA, TNakao, SM, YI, MW, TNarita, HI, MM, and TS; supervision, MH, MM, and TS; project administration, HT, MH, MM, and TS; funding acquisition, MH, HI, MM, and TS. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

APC	adenomatous polyposis coli
ASA	aspirin, acetylsalicylic acid
5-ASA	mesalazine, 5-aminosalicylic acid
CCK-F	Cell Counting Kit-F
CDK	cyclin-dependent kinase
CI	combination index
CM	colon mucosa cells
COX	cyclooxygenase
CP	colonic polyp-derived cells
CRC	colon cancer
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DSS	dextran sulfate sodium
FAP	familial adenomatous polyposis
FBS	fetal bovine serum
qRT-PCR	quantitative reverse transcription-PCR
SIP	small intestinal polyp-derived cells
SS	sodium salicylate
TNF-α	tumor necrosis factor α
UC	ulcerative colitis

Conflict of Interest

No potential conflicts of interest were disclosed.

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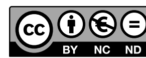
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