Original Article

Chemoprevention by aspirin against inflammation-related colorectal cancer in mice

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Inflammation is a primary risk factor for cancer. Epidemiological studies previously demonstrated that aspirin decreased the incidence of cancer and specifically reduced the risk of colorectal cancer. However, the number of animal studies that have confirmed the efficacy of aspirin remains limited. Therefore, the purpose of the present study was to investigate the mechanisms by which aspirin prevents colorectal cancer in mice. ICR mice were treated with azoxymethane and the ulcerative colitis inducer, dextran sodium sulfate, to induce colorectal tumors. Aspirin was orally administered three times per week for 12 weeks. Aspirin significantly reduced the number and size of colorectal tumors. Aspirin also significantly decreased tumor necrosis factor alpha and reactive oxygen species (ROS) levels in the plasma. Immunohistochemical analyses and western blots showed that cyclooxygenase 2 (COX2), inducible nitric oxide synthase (iNOS), and the active form of Yes-associated protein 1 (YAP1), and cytosolic high mobility group box 1 (HMGB1) were strongly expressed at colorectal tumor sites and clearly suppressed by aspirin. An indicator of inflammation-related DNA damage, 8-nitroguanine, also accumulated in the colorectal tissues and was suppressed by aspirin. The present results suggest that the ingestion of aspirin suppressed carcinogenesis caused by inflammation through decreases in COX2 and ROS levels, resulting in reductions in DNA damage and oncogenic YAP1.

Key Words: **inflammation, reactive oxygen species, cyclooxygenase 2, yes-associated protein 1, 8-nitroguanine**

Chronic inflammation is a primary risk factor for the development and progression of cancer, and has been implicated in approximately 25% of human cancer cases worldwide.[1,2] Epidemiological studies demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, decreased the incidence of cancer, and specifically reduced the risk of colorectal cancer.[3–8] The reliable approach of chemoprevention may come out through an elucidation of the mechanism. The mechanism for cancer prevention by aspirin may be explained by anti-inflammatory effect as an inhibitor of cyclooxygenase (COX). Recently, many other targets such as WNT/β-catenin signalling, platelet, AMP-activated protein kinase, cyclin-dependent kinase, heparanase, and histone have been reported to relate to anti-cancer effect of aspirin.[9,10] However, the mechanism is not fully clarified.

Inflammatory bowel diseases (IBD) are associated with colorectal cancer.[11,12] In research on the mechanisms underlying inflammation-related colorectal carcinogenesis, azoxymethane (AOM)/dextran sodium sulfate (DSS) mice are widely used as a colitis-associated colorectal cancer model, and tumors are induced by the chemical carcinogen, AOM, with an inflammatory stimulus by DSS.[13,14] This mouse model develops colitis and pathological findings that mimic those of IBD in humans. The present study examined the suppressive effects of aspirin on the incidence of tumors in AOM/DSS mice, and investigated the mechanisms responsible for its chemopreventive effects.

Materials and Methods

Ethics. The present study was performed in accordance with the recommendations by the Guide for the Care and Use of Laboratory Animals of Suzuka University of Medical Science (approval number: 71).

Animals. Specific pathogen-free female ICR mice (7 weeks old) were purchased from SLC (Hamamatsu, Shizuoka, Japan) and individually housed in cages at 23 ± 1°C in an air-conditioned room with controlled humidity (59% ± 10%) and a 12-h light/dark cycle. Each group comprised four or five mice. Experiments were performed in duplicate. Surgery was conducted using pentobarbital anesthesia, and all precautions were taken to minimize suffering.

Experimental design. Mice were quarantined in the first week and then randomly divided by body weight into the following groups: no treatment, aspirin, AOM/DSS + water, and AOM/DSS + aspirin. The AOM/DSS + water and AOM/DSS + aspirin groups were intraperitoneally administered a single injection of AOM (10 mg/kg body weight; Sigma Chemical Co., St. Louis, MO); 1 week after the injection, these groups were treated with 4% DSS (molecular weight of 36,000–50,000; MP Biomedicals, Inc., Solon, OH) in their drinking water for 7 days, followed by regular water to the endpoint. Acetylsalicylic acid (100 mg/kg body weight; Sigma Chemical Co.) dissolved in sodium carbonate solution was orally administered to the aspirin and AOM/DSS + aspirin groups using a feeding needle three times per week for 12 weeks. Water was administered to the AOM/DSS + water group instead of the aspirin solution.

Histopathological study. Colon tissue and blood samples were collected 12 weeks after the initiation of the experiment. The large bowel (from the ileocecal junction to the anal verge) was measured, opened longitudinally along the main axis, and then washed with PBS. After macroscopic inspection, the bowel was cut, fixed with formalin, and embedded in paraffin. Histopathological changes were assessed in paraffin sections stained with hematoxylin and eosin (HE), according to the standard method. Benign and malignant areas were histopathologically distinguished using HE-stained tissue sections by two investigators. Colorectal tumors were detected in six out of the nine mice in the AOM/DSS + water group and five out of the nine mice in the AOM/DSS + aspirin group.

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Immunohistochemical study. The following primary antibodies were used: a rabbit polyclonal anti-cyclooxygenase 2 (COX2) antibody (ab52237; Abcam plc, Cambridge, UK) 1:200, anti-inducible nitric oxide synthase (iNOS) antibody (ab15323; Abcam plc) 1:100, a rabbit monoclonal anti-active (non-phosphorylated) form of the Yes-associated protein 1 (YAP1) antibody (EPR19812, ab205270; Abcam plc) 1:1,000, and an anti-high mobility group box 1 (HMGB1) antibody (ab79823; Abcam plc) 1:500. A rabbit polyclonal anti-8-nitroguanine antibody without a cross-reaction was produced as described previously.[15] Immuno reaction was performed using the VECTASTAIN® Elite ABC kit (Funakoshi Co., Ltd. Tokyo, Japan), in accordance with the manufacturer's instructions. Then the reacted sections were stained with a Peroxidase Stain DAB Kit (Brown Stain) (Nacalai Tesque, Inc., Kyoto, Japan) and counterstained with hematoxylin.

The semi-quantitative analysis of staining intensities was graded using IHC scores of 0–4 by two investigators in all IHC studies as follows: no staining (0), ambiguous staining (0.5), weak staining (1+), moderate staining (2+), strong staining (3+), and very strong staining (4+). Normal sites were analyzed for all mice, i.e., eight mice each for the no treatment and aspirin groups, and nine mice each for the AOM/DSS + water and AOM/DSS + aspirin groups. Malignant sites were analyzed for all mice with colorectal tumors, i.e., six mice in the AOM/DSS + water group and five mice in the AOM/DSS + aspirin group.

Plasma assay. Blood samples were collected from mice on the final day of each experimental group. Blood samples from four mice in each group of each experiment were used for analyses. Plasma interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) in accordance with the manufacturer's instructions.

Assay of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Blood samples and colonic tissue samples were collected from mice on the final day. For analyses of ROS/RNS in plasma, blood samples from four mice in each group of an experiment were used. For analyses of ROS/RNS in colon, diluted solutions extracted from tissue samples of three mice in each group of another experiment were used. Protein concentrations of tissue samples were equalized by the bradford assay (Bio-Rad DC™ Protein Assay; Bio-Rad Laboratories, Inc.). The levels of ROS/RNS in tissue samples were shown as relative ratio vs mean value of "No treat" group. ROS/RNS levels were assessed using the OxiSelect™ In Vitro ROS/RNS Assay Kit (STA-347; Cell Biolabs, Inc., San Diego, CA) in accordance with the manufacturer's instructions.

Western blotting analysis. Colon tissue samples were collected 12 weeks after the initiation of the experiment. The tissues were immediately put into liquid nitrogen and stored at −80°C until use. The frozen tissues were homogenized in lysis buffer (Kurabo, Osaka, Japan), centrifuged at 8,000 *g* for 20 min at 4°C, and supernatants were collected. Protein concentrations were determined by the bradford assay. SDS-PAGE was performed as previously described.[14] The membranes were incubated at room temperature for 1 h with primary antibodies against COX2 (12282; Cell Signaling Technology) 1:1,000, iNOS (ab178945; Abcam plc) 1:1,000, YAP1 (ab205270; Abcam

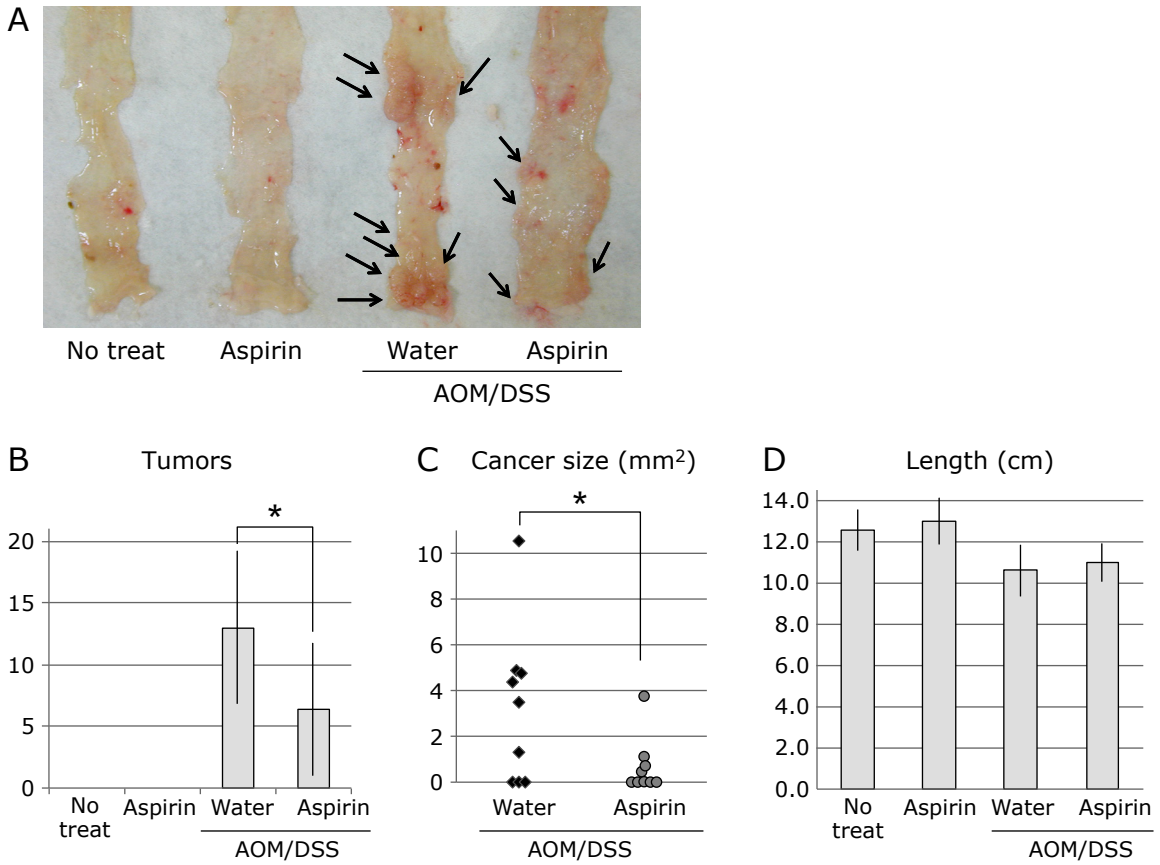

Fig. 1. Suppressive effects of aspirin on colorectal carcinogenesis in mice treated with AOM and DSS. (A) Macroscopic view of colorectal tumors (indicated by arrows), (B) numbers of colorectal tumors per mouse, (C) microscopic analysis of cancer area with malignant cells per mouse in tissue sections, and (D) large bowel lengths. The area of malignant cells was determined by microscopic analysis of HE-staining sections. The results were obtained from duplicate experiments. Eight mice each for the no treatment and aspirin groups and nine mice each for the AOM/DSS + water and AOM/DSS + aspirin groups were used. *p<0.05.

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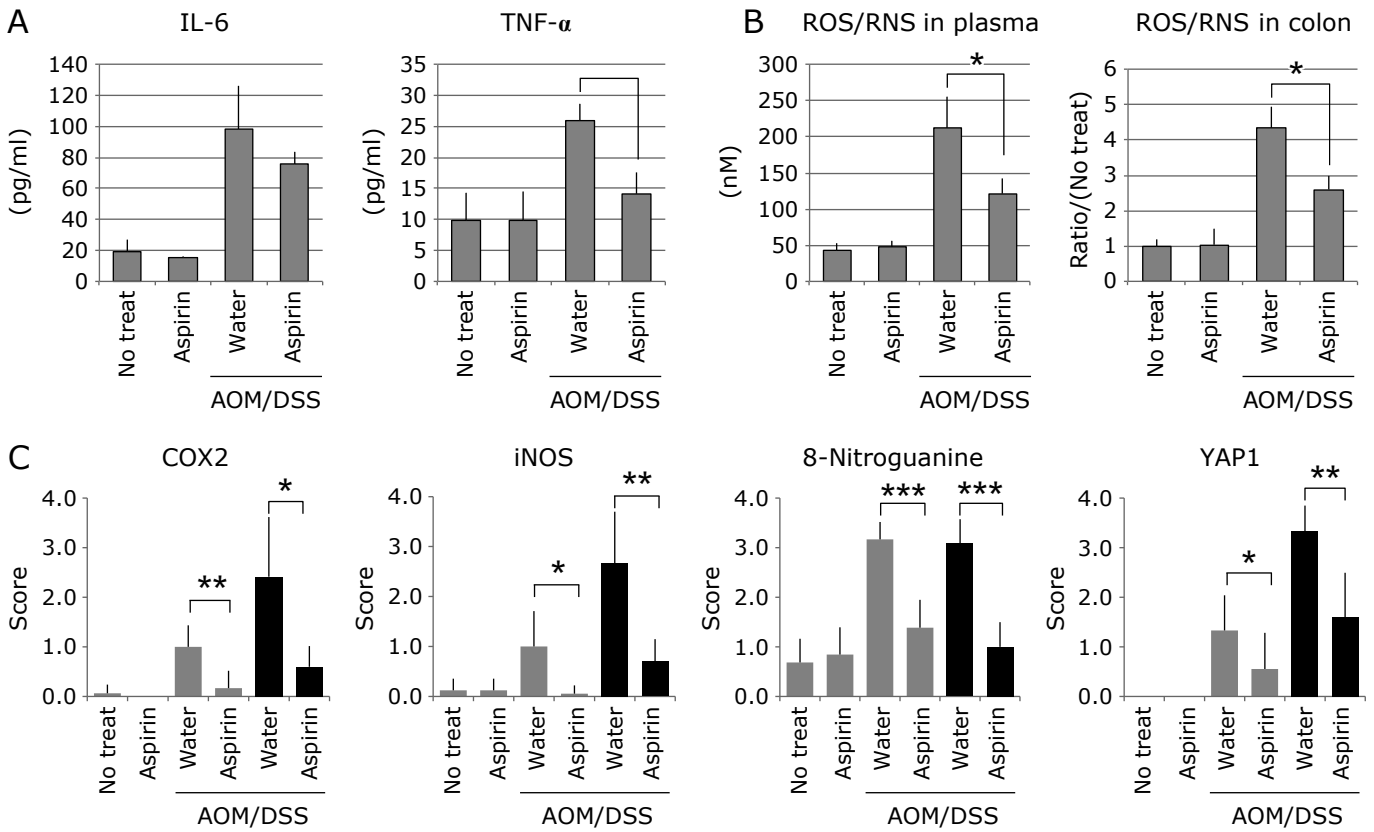

Fig. 2. Suppressive effects of aspirin on markers related to inflammatory stress and cancer. (A) Plasma levels of IL-6 and TNF-α ($n = 4$). (B) ROS/RNS levels in plasma and in colonic tissue. The results of plasma assay were obtained from four mice for each group. The results of tissue assay were obtained from three mice of another experiment for each group. The levels of ROS/RNS in tissue samples were shown as relative ratio vs mean value of "No treat" group ($n = 3$). (C) Score of immunohistochemistry. Gray bars are normal sites and black bars are malignant sites of colorectal tissues from mice. The results of normal sites were obtained from all mice of duplicate animal experiments ($n = 8$ for groups without AOM/DSS, $n = 9$ for groups treated with AOM/DSS). The results of malignant sites were obtained from mice with colorectal cancer ($n = 6$ for AOM/DSS + water, $n = 5$ for AOM/DSS + aspirin). Graphs represent the average score (bar; SD). *p<0.05, **p<0.01, ***p<0.001 between water and aspirin groups by Student's t test.

plc) 1:1,000, HMGB1 (ab79823; Abcam plc) 1:1,000, and β-actin as a loading control (58169; Cell Signaling Technology) 1:5,000. The immune complex on the membranes was visualized using horseradish peroxidase-conjugated secondary antibody (Novex, Frederick, MD) and detected with ImmunoStar Zeta reagent (Wako, Osaka, Japan). The images of the membranes were acquired using a multi-grade software program (Fuji-film, Greenwood, SC).

Statistical analysis. Data are shown as means with SD. Comparisons of data between groups were performed using the Student's t test. A p value of less than 0.05 was considered to be significant.

Results

Suppressive effects of aspirin on colorectal cancer induced by AOM and DSS. Blood and colorectal tissue samples were collected following the completion of the 12-week treatment, and the macroscopic appearance of colorectal tumors was examined. Tumor malignancy was microscopically observed using HE-stained tissue sections. Aspirin attenuated the symptoms of colorectal cancer (Fig. 1A–C). The oral administration of aspirin significantly decreased the number of colorectal tumors per mouse, i.e., 13.0 ± 6.2 in the water-treated group and 6.3 ± 5.4 in the aspirin-treated group (p<0.03, $n = 9$). The administration of aspirin also significantly decreased the area of malignant cells in AOM/DSS mice ($p = 0.030$, $n = 9$). Mean large bowel lengths are shown in Fig. 1D. Large bowel lengths were shorter in the AOM/DSS-treated groups than in the control groups. No significant changes were observed in large bowel lengths between the aspirin- and water-treated groups.

Levels of IL-6, TNF-α, and ROS/RNS. The plasma levels of the inflammatory mediators IL-6 and TNF-α were assessed using blood samples (Fig. 2A). Plasma IL-6 levels were slightly lower in the AOM/DSS + aspirin group than in the AOM/DSS + water group, while plasma TNF-α levels were significantly decreased in the AOM/DSS groups by aspirin ($p = 0.0038$, $n = 4$). The production of ROS/RNS was enhanced under inflammatory conditions (Fig. 2B). The ROS/RNS levels were significantly decreased in the AOM/DSS groups by aspirin (plasma; $p = 0.017$, $n = 4$, colon; $p = 0.015$, $n = 3$). The alterations of the levels of ROS/RNS showed similar tendencies in plasma and in colon, suggesting that serum ROS/RNS derived from colon with inflammation.

Immunohistochemistry of colorectal tissues. A summary of data obtained from the immunohistochemical analysis is shown in Fig. 2C and representative images in Fig. 3. The intensities of immunostaining for COX2, iNOS, inflammation-related DNA damage, 8-nitroguanine, non-phosphorylated active YAP1, and HMGB1 were significantly weaker in the AOM/DSS + aspirin group than in the AOM/DSS + water group (Fig. 2C). As shown in Fig. 3, COX2 and iNOS immunoreactivities were weakly observed in the cytoplasm of colonic epithelial cells on colonic lumen surface, as compared to the no staining in No treat

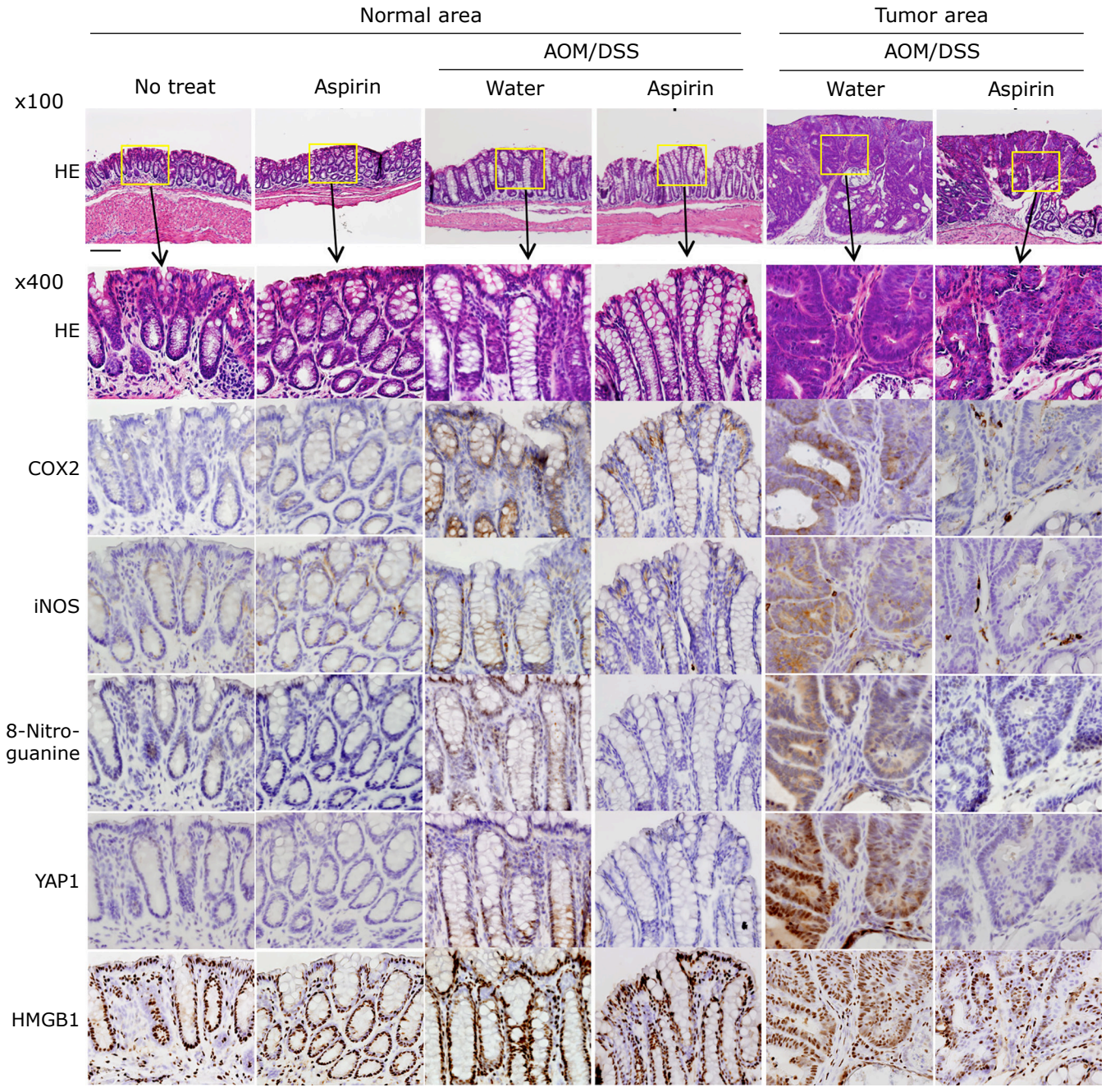

Fig. 3. Histopathology and immunohistochemistry of colorectal tissues. Aspirin depressed COX2, iNOS, 8-nitroguanin, YAP1, and cytosolic HMGB1 at tumor sites of colorectal tissues of mice. 8-Nitroguanine accumulated at the cancerous sites. HMGB1 spread to cytosol from nucleus at cancerous sites, which was suppressed by aspirin. Representative images are shown (Original magnifications, ×100 and ×400. Scale bars = 100 μM).

and Aspirin group in the normal area of colon. Tumor section shows strongly COX2 and iNOS immunoreactivities in the colon cancer cells in the AOM/DSS + water group that theirs in the AOM/DSS + aspirin group. 8-Nitroguanine was observed at the nucleus in normal area of the AOM/DSS + water group, and strongly in the colon cancer cell in tumor area of the AOM/DSS + water group. Active YAP1 was weakly detected at the base of colonic mucosal epithelium cells in normal area of the AOM/DSS + water group, and strongly detected in the cancer cell colorectal tumor area in the AOM/DSS + water group. HMGB1, which plays a role in inflammatory signals, was observed in the nucleus of cells in normal area of all mice colonic epithelial cells, and in tumor area of the AOM/DSS + aspirin group. The translocated HMGB1 was observed in the cytoplasm expression that shift from nucleus at malignant tumor cell in the AOM/DSS + water group.

Western blotting analysis. The expressions of COX2, iNOS, YAP1, and HMGB1 in cytosol in colon tissues were shown in Fig. 4B and representative images in Fig. 4A. The levels of COX2, iNOS, YAP1, and HMGB1 were high in the AOM/DSS + water group, and significantly attenuated in the AOM/DSS + aspirin group ($p = 0.019$ for COX2, 0.0026 for iNOS, 0.0002 for YAP1, and 0.0015 for HMGB1).

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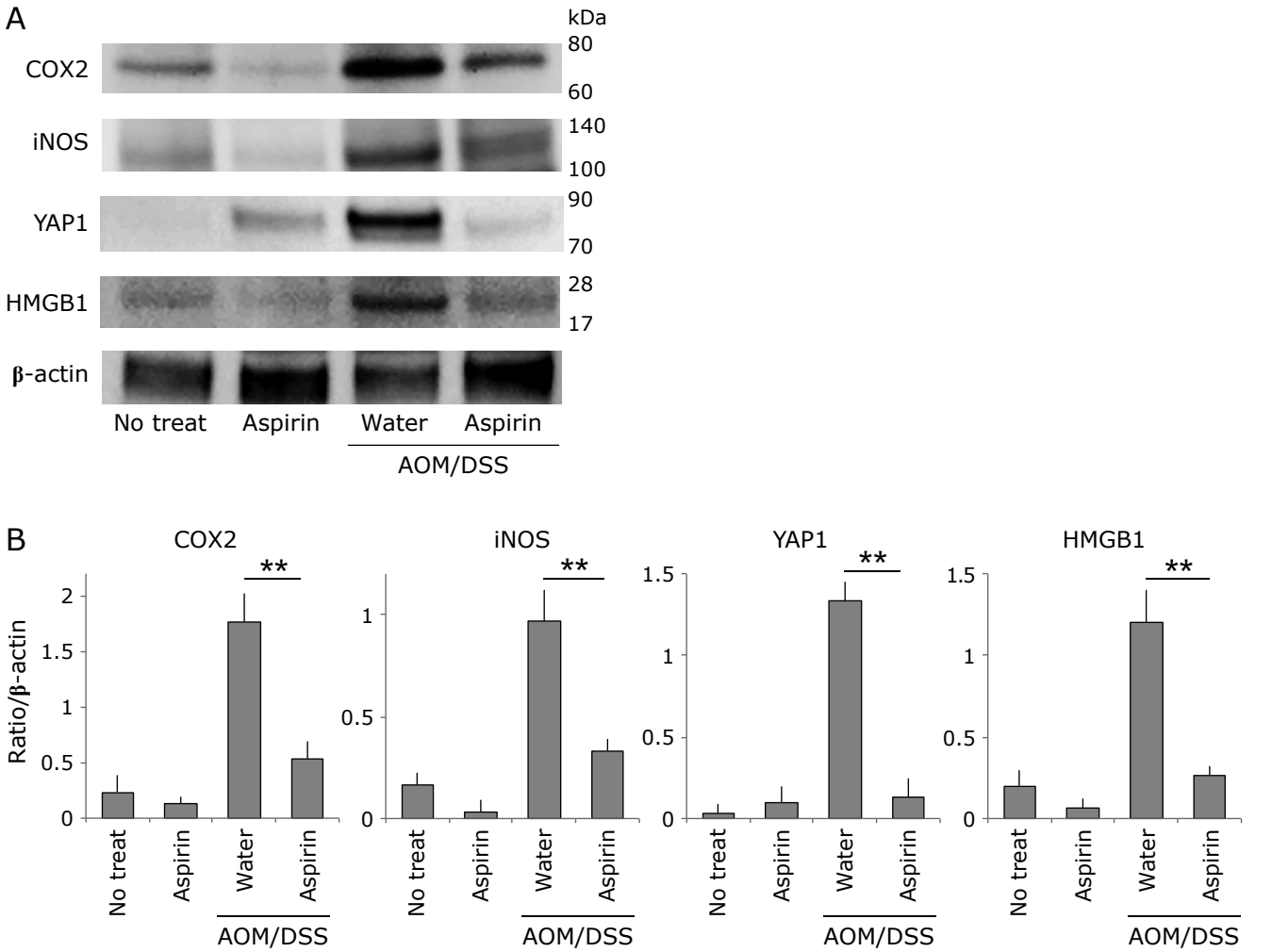

Fig. 4. Western blotting analysis of COX2, iNOS, YAP1, and HMGB1 in colon tissues. (A) Representative images. (B) Ratio of expression levels of COX2, iNOS, YAP1, and HMGB1 vs β-actin. Graphs represent the average score ($n = 3$, bar; SD). $^{**}p<0.01$; between water and aspirin groups by Student's *t* test.

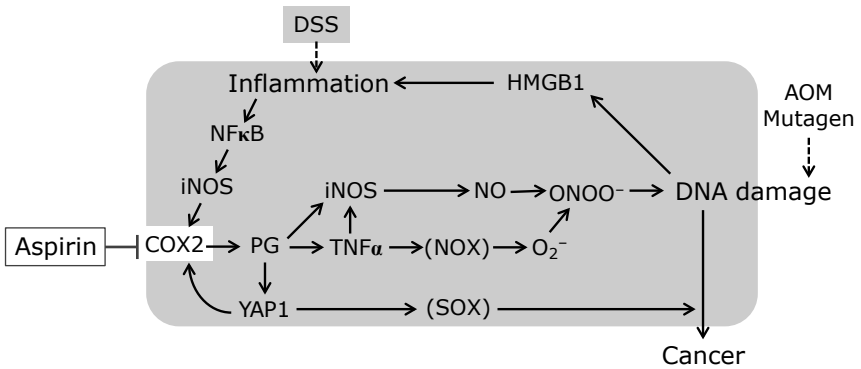

Fig. 5. Possible mechanism of cancer prevention by aspirin.

Discussion

This study is the first report to demonstrate that oral administration of aspirin significantly reduced inflammation-related DNA damage and colorectal cancer incidence in AOM/DSS mice. It was also shown that aspirin significantly reduced COX2, iNOS, ROS/RNS, 8-nitroguanine, YAP1, and TNF-α in AOM/DSS mice. IL-6 reduction was statistically not significant, but Fig. 2 shows similar tendency to decrease as other markers of inflammation-related carcinogenesis. On the basis of these results and the related papers, we proposed the possible mechanism of inflammation-related carcinogenesis (Fig. 5). Inflamma-

tion induces NFκB/iNOS/COX2-axis. Then, COX2-mediated prostaglandin (PG) E2 increases YAP1.[16] Yes-associated protein (YAP) is a transcriptional co-activator and a major effector of the Hippo pathway.[17] YAP is thought to promote cancer initiation and progression, because it promotes cell proliferation and stemness.[18] COX2 and YAP1 regulate each other. COX2 is a stimulus of YAP and a target of Hippo/YAP pathway, thus forming a positive feedback circuit.[19] It has been reported that co-operation of COX2 and YAP1 recruit SOX2,[20] which is cancer stem cell marker and promotes malignancy.[21,22] YAP1 is also reported to up-regulate SOX9,[23–26] SOX5[27] in relation to malignancy of various cancers. The transcriptional regulator YAP plays an important role in cancer progression and frequently overexpressed in human cancers.[28,29] YAP1 has oncogenic properties and correlates with the prognosis of colorectal cancer patients.[30,31] Therefore, it is important that aspirin suppresses not only COX2 but also YAP1 in colorectal tumors in mice.

Nitric oxide (NO) and ROS are reported as potential mediators of the epithelial-mesenchymal and mesenchymal-epithelial transitions in cancer development.[32] Under inflammatory conditions, NO and superoxide (O_2^-) are released from neutrophils or macrophages during inflammation. NO is generated by iNOS in inflammatory cells and in epithelial cells via NFκB and PG.[33] NO can diffuse through the extracellular matrix, cross the plasma membrane and the cytoplasm of epithelial cells, and enter the nucleus. TNF-α stimulates O_2^- via the induction of NAD(P)H oxidase (NOX) in epithelial cells.[34] NO reacts with O_2^- to form peroxynitrite, which causes nitrative DNA damage to generate 8-nitroguanine.[35,36] 8-Nitroguanine can cause mutations and has been implicated in inflammation-mediated carcinogenesis.[35,36] In this study, 8-nitroguanine was accumulated in colorectal tissues of mice with DSS treatment, but not in those without DSS treatment. Furthermore, the accumulation of 8-nitroguanine was decreased by administration of aspirin. Therefore, inflammation-related DNA damage plays significant role in the colitis-related carcinogenesis, in addition to DNA damage by chemical mutagen AOM.

8-Nitroguanine was accumulated in tumorigenic sites more than in normal sites of AOM/DSS mice. Relevantly, we also reported that 8-nitroguanine and iNOS were observed in colon epithelium of another model of IBD, which was induced by transfer of $CD45RB^{high}CD4^+$ T cells lacking regulatory T cells to severe combined immuno- deficiency mice.[37] In malignant tumors, the cellular localization of HMGB1 tended to change to cytosol from nucleus (Fig. 3). Amounts of HMGB1 in cytosol were increased in colon of AOM/DSS mice and attenuated by aspirin (Fig. 4). The localization of HMGB1 may be important character in inflammation-related carcinogenesis. HMGB1, which is released from damaged cells, can promote inflammatory signals, leading to inflammation-related DNA damage such as 8-nitroguanine and carcinogenesis.[33,38] Interestingly, this study shows inflammation-related risk continuously occurs in colorectal tissues after 10 weeks of DSS treatment. Inflammation may be induced by other risk factors such as DNA damage. These suggest that inflammation both by DNA damage as well as by DSS plays important roles on colitis-related colorectal cancer.

In conclusion, aspirin may contribute to prevent inflammation-related carcinogenesis by reducing COX2 and YAP1.

Author Contributions

SO conceived of the hypothesis, designed and performed experiments, interpreted results, and wrote the manuscript. KH performed experiments and participated in critical discussions. NM provided pathology expertise and participated in critical discussions. SK participated in critical discussions, helped edit the manuscript, and supervised the project.

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Abbreviations

AOM	azoxymethane
COX2	cyclooxygenase 2
DSS	dextran sodium sulfate
HE	hematoxylin and eosin
HMGB1	high mobility group box 1
IBD	inflammatory bowel diseases
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
NSAIDs	non-steroidal anti-inflammatory drugs
PG	prostaglandin
RNS	reactive nitrogen species
ROS	reactive oxygen species
TNF-α	tumor necrosis factor alpha
YAP1	Yes-associated protein 1

Conflict of Interest

No potential conflicts of interest were disclosed.

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