Inhibition of chronic ulcerative colitis associated adenocarcinoma development in mice by inositol compounds

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Chronic inflammation is a well recognized risk factor for cancer and patients with long-standing ulcerative colitis (UC) are at an increased risk for colorectal carcinoma development. In order to prevent UC associated carcinogenesis, we tested the effects of inositol compounds (including inositol and hexaphosphate inositol) on UC-associated carcinogenesis in our novel mouse model. Female C57BL/6 mice were subjected to long-term, cyclic dextran sulfate sodium (DSS) treatment and fed a 2-fold iron-enriched diet. The inositol compounds were administered via the drinking fluid. In the DSS-plus-2-fold iron positive control group, colorectal adenocarcinoma incidence was 70.6% (24/34 mice) after 15 cycles of DSS treatment (1 DSS cycle = 7 day DSS treatment period followed by a 10 day recovery period). Tumor multiplicity was 1.26 ± 1.05 and tumor volume was 21.4 ± 5.2 mm3. Adding 1% inositol, tumor incidence was statistically significantly reduced (42%, 9 of 21 mice with tumors), as was tumor multiplicity (0.5 ± 0.7) and tumor volume (4.2 ± 1.9 mm3). Administration of hexaphosphate inositol noticeably reduced tumor incidence (50%, 12 mice with tumors out of 24 total), tumor multiplicity (0.8 ± 0.9) and tumor volume (12.3 ± 4.1 mm3); however, the results were not statistically significant (P > 0.05). Further mechanistic studies showed that the inhibition of UC-associated carcinogenesis by inositol compounds might relate to their function on the modulation of macrophage mediated inflammation, nitro-oxidative stress and cell proliferation in UC-associated carcinogenesis. This study indicates that inositol compounds may have the potential to serve as preventive agents for chronic inflammation-carcinogenesis.

Introduction

Myo-inositol is the head group of polyphosphate inositol (1). Phosphatidylinositol is the most abundant phosphoinositide in mammalian cells and they participate in a variety cellular functions (1–6). The metabolism of these phosphoinositotides is highly active and accurately controlled (1,6,7); they can undergo sequential and reversible phosphorylations by specific kinases and phosphatases (1). The metabolites are involved in the regulation of several conventional enzyme activities and protein phosphorylation in the cell membrane, cytoplasm and nucleus, including casein kinase 2 and G-protein family, chromatin re-modeling and DNA repair, phospholipase, protein kinase C, etc. (4–6,8–11). The most well-known metabolites, IP2 and IP3, and their associated PI 3-kinase/PTEN pathways play critical roles in cell proliferation and carcinogenesis (10,12). Thus, this raises the important question of whether dietary inositol compounds (nutrients) are involved in the regulation of cellular activities and in the modulation of disease processes, such as inflammation-driven carcinogenesis.

Epidemiological studies and experimental evidence have suggested an inverse relationship between colon cancer and high-fiber foods (13–19). Polyphosphate inositol compounds [Inositol hexaphosphate (IP6)] are the most active components in high-fiber foods (18,20). IP6 is abundant in whole grains, cereals, legumes, nuts and seeds as the primary energy source and antioxidant for the germinating plant (1). Anti-neoplastic activity by IP6 and inositol has been extensively studied by Shamsuddin, Lee Wattenberg, and others (20–26). In vivo studies in chemical carcinogen-induced tumor models in rodents reveal that inositol compounds inhibit neoplastic growth and development in various organs including the colon, lung, breast, prostate, liver, skin and soft tissues (20,25–29). Studies of molecular mechanisms mainly from in vitro cell culture models show that the anti-cancer activity of inositol compounds may result from the modulation of cell signal transduction, inhibition of cell proliferation and arrest of cell cycle progression, induction of cell differentiation, increases in natural killer cell activity and anti-oxidant activity (20,30–38). Translation of in vitro results into in vivo systems remains to be investigated. There has yet to be a study testing the effects of inositol compounds on suppressing chronic inflammation-driven carcinogenesis.

Long-standing chronic inflammation is a well recognized risk factor for the development of cancer in various organs (39–41). Ulcerative colitis (UC) is a typical chronic inflammatory bowel disease conferring an increased risk for colorectal cancer (42). Inflammation-caused nitrative and oxidative stress due to the overproduction of reactive oxygen and nitrogen species (RONS) and a depletion of antioxidant molecules play crucial roles in chronic inflammatory activity and inflammation associated carcinogenesis in UC patients (43–48). RONS may contribute to the initiation and promotion of carcinogenesis in the UC setting through mutagenic and pro-mitogenic activities (42,49). Cell proliferation is required for mutagenesis. Hyper-proliferative status due to inflammation-induced overproduction of inflammatory mediators, (such as prostaglandins), cytokins and growth factors also increases susceptibility to mutagenesis and promotes carcinogenesis.
Long-standing inflammation associated anemia (mainly due to iron deficiency) is another complication in UC patients who are encouraged to take foods enriched in iron. To study the association of a high iron diet with UC-associated carcinogenesis, a novel UC-associated carcinogenesis mouse model was established in our laboratory (50). The oral administration of dextran sulfate sodium (DSS) to rodents induces colonic mucosa inflammation that is clinically and histologically similar to human UC (51). Like in humans, DSS-induced chronic UC is complicated by the development of colorectal dysplasia and adenocarcinoma, but has a low tumor incidence (50,52). In our studies, 2-fold dietary iron supplementation in DSS-induced long-term chronic UC mice significantly enhanced colorectal carcinoma development with >70% tumor incidence (50). This DSS-induced and iron-enhanced UC-associated carcinogenesis mouse model, without the use of chemical carcinogens, provides an excellent model system to study the mechanism and prevention of inflammation-driven carcinogenesis.

In the present studies, the effects of inositol and IP6 on UC-associated carcinogenesis were studied using a DSS-induced and iron-enhanced UC-associated carcinogenesis model in mice. Further mechanistic studies on macrophage-mediated inflammation, cell proliferation and nitro-oxidative stress were analyzed immunohistochemically.

Materials and methods

Animals

Six-week-old female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were housed five per cage under pathogen-free conditions in the facilities of Laboratory Animal Services, with a university approved protocol. All mice were acclimated to animal room conditions for two weeks before the start of the experiment, during which time they were administered ordinary lab chow and tap water ad libitum. Body weights and food consumption were monitored every other week for the duration of the experiment.

Chemicals and diet

DSS (MW: ~40 000) was purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Mayer’s hematoxylin solution, eosin Y solution and 3,3′-diaminobenzidine (DAB) were from Sigma Chemical Co. (St. Louis, MO). Antigen unmasking solution (AUS) and avidin/biotinylated horseradish peroxidase (HRP) macromolecular complex kits were from Vector Laboratories (Burlingame, CA). Polyclonal anti-Mac3 (specific to mouse macrophages) was from BD Pharmingen (San Diego, CA). Polyclonal anti-iNOS (anti-NOS2, antibody M-19) antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal anti-nitrotyrosine antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-proliferating cell nuclear antigen (PCNA) was from Oncogene Science, Inc. (San Diego, CA). AIN76A-based diet containing 45 mg iron per kg diet was purchased from Research Diets, Inc. (New Brunswick, NJ). Myo-inositol and hexaphosphate inositol were purchased from Sigma.

Animal experiment design

In a long-term study of chronic UC and associated colorectal carcinogenesis, mice were randomized into six groups. Group 1, Group 2 and Group 3 were administered water, 1% inositol and 1% hexaphosphate inositol, respectively in the drinking water throughout the experiment as negative controls (n = 5 mice per group). Group 4 (n = 40 mice), Group 5 and Group 6 mice (n = 25 mice per group) were subjected to cyclic DSS treatment, as per our previously established methods (50). One DSS ‘cycle’ consisted of 7 days of 1% DSS (w/v in deionized water) through the drinking fluid (DSS period), followed by 10 days of ordinary tap water (recovery period). More animals were assigned to Group 4 in order to prevent a marked decrease in animal numbers at the end of experiment possibly due to severe UC.

The negative and positive control groups were given deionized water during the DSS period and ordinary tap water during the recovery period. The mice were subjected to 15 consecutive DSS cycles. Starting 2 days before the start of the experiment and continuing for the duration of the experiment, all groups were given a modified AIN76A diet containing 90 mg iron per kg diet (AIN76A/2XFe diet; purchased from Research Diets, Inc., New Brunswick, NJ). Groups 5 and 6 mice were administered 1% inositol or 1% hexaphosphate inositol, respectively in the drinking fluid during the recovery period. The mice were sacrificed at the end of the 15th DSS cycle (Day 255). Mice were sacrificed before the end of the experiment period if they exhibited significant weight loss (>20%), excessive rectal bleeding and loss of activity. Mice sacrificed before the completion of 15 DSS cycles were excluded from the results of the carcinogenesis experiment.

Tissue preparation and histopathological evaluation

After sacrificing the mice by CO2 asphyxiation, the colons were perfused with normal saline, removed and opened longitudinally. The numbers and locations of colon tumors were recorded, and three perpendicularly diameter measurements were obtained for each tumor using calipers. Tumor volume was calculated using the formula for a sphere (π/6 × radius3) and the average tumor radius obtained from the three diameter measurements. The colons were then fixed in 10% formalin for 24 h, routinely processed and embedded in paraffin as a ‘Swiss roll’. Serial tissue sections (5 μm) were made and stained with hematoxylin and eosin (H & E) for histopathological analysis, or used for immunohistochemical staining.

Colorectal tumors were analyzed by light microscopy and classified as colorectal adenocarcinoma according to established criteria (50). The tumors were further categorized as tubular, well-differentiated adenocarcinoma or mucinous adenocarcinoma based on the glandular architecture and presence or absence of mucin lakes (>75% of cancer nests with mucin overproduction).

Histological grading of UC

H & E stained serial tissue slides were used for histological scoring of the UC index in the colon. As compared to the colons of negative control mice (morphologically normal mucosa), the colons of mice treated with DSS/2XFe showed both non-actively-inflamed mucosa (showing mild hyperplastic change with glandular crypt distortion, basal plasmacytosis and frequent lymphoid aggregates) and actively-inflamed mucosa (showing erosion, ulceration and cryptitis with epithelial injury and reactive epithelial changes). The UC index was obtained from the scores for inflammation severity, ulceration, hyperplasia and area of inflammatory involvement. The criteria for the scoring of these parameters have been described previously (50,53). The mean score for each parameter was obtained from the individual scores for 5 to 6 mice per group. The total score (UC index) was the sum of the individual parameter scores for each mouse. The mean UC index for each group was the average of the total scores for 5 to 6 mice per group.

Immunohistochemistry

Immunohistochemical staining was performed using the avidin–biotin–peroxidase complex method as described previously (50,54). In brief, endogenous peroxidase activity was quenched in paraffin embedded tissue sections with 1% H2O2. After retrieving the antigen by pretreatment with a citrate buffer (AUS, Vector Laboratories, Burlingame, CA) in a microwave oven and blocking non-specific protein-binding interactions with diluted normal serum, the slides were incubated with the primary antibody (5 μg/ml for anti-Mac3, 1:1000 dilution for polyclonal anti-nitrotyrosine and monoclonal anti-PCNA), followed by the appropriate biotinylated secondary antibody and the ABC complex for 45 min each. Diaminobenzidine was used as the chromagen. Slides were washed thoroughly between incubations with phosphate-buffered saline (PBS). Negative controls were established by replacing the primary antibody with PBS and normal serum. Positive staining was indicated by the presence of a brown-colored precipitate.

Assessment of Mac3-staining macrophage and nitrotyrosine formation

The mucosal infiltration of macrophages and nitrotyrosine formation were assessed by immunohistochemical staining anti-Mac3 and anti-nitrotyrosine antibodies. The densities of Mac3-positive cells or nitrotyrosine-positive cells in the colonic mucosa were defined as the number of immunostained-positive cells per mm2 of mucosa. Positive cell numbers and mucosal and tumor areas were measured using a Nikon research microscope in conjunction with a Nikon DXM 1200 digital camera and image analysis software (Image-Pro Plus Version 4.5, Media Cybernetics, Silver Spring, MD). The immunostained-positive cell density was determined in six microscope fields (10× objective lens) of the distal colon or tumor per mouse. Slides from five randomly chosen mice per group were analyzed.

PCNA labeling index

Immunohistochemistry for PCNA was used as a measurement of cell proliferation. The PCNA labeling index was determined for the epithelia in colorectal dysplasia and adenocarcinoma, but has a low tumor incidence (50,52). In our studies, 2-fold dietary iron supplementation in DSS-induced long-term chronic UC mice significantly enhanced colorectal carcinoma development with >70% tumor incidence (50). This DSS-induced and iron-enhanced UC-associated carcinogenesis mouse model, without the use of chemical carcinogens, provides an excellent model system to study the mechanism and prevention of inflammation-driven carcinogenesis.

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non-inflamed mucosa and inflamed mucosa in the distal colon and for tumors (most of the inflammatory activity occurred in the distal colon; within a 3 cm segment of the colon from the anus). The proliferation index was defined as the ratio of the number of PCNA immunostained-positive cells and the total number of epithelial or tumor cells counted. Slides from 6 mice per group were analyzed and greater than 1000 epithelial cells or tumor cells were counted per slide.

Statistical analysis
The data were expressed as the mean ± SE. Statistical significance was tested using SigmaStat Version 1.01 software (Jandel Scientific, San Rafael, CA). Tumor incidence was analyzed using the χ² test. Normally, distributed data were analyzed using Student’s t-test and non-parametric data, such as tumor multiplicity, were analyzed using the Mann–Whitney Rank Sum test. P-values less than 0.05 were taken to be statistically significant.

Results

Body weight, food consumption and mortality
In this long-term animal experiment, in negative control groups (Groups 1, 2 and 3), the average body weight of Group 1 (water control) mice did not differ from that of Groups 2 and 3 (inositol and IP6 control groups). At the end of the experiment, the body weights were 22.8 ± 1.9 g, 23.2 ± 1.7 g and 22.6 ± 1.9 g, respectively (mean ± SE). In DSS-induced UC groups (Groups 4, 5 and 6), the average body weights for Group 4 (DSS-treatment positive control group) at the end of the experiment was 21.9 ± 1.9 g. In Inositol (Group 5) and IP6 (Group 6) treatment groups, the body weights were 21.4 ± 1.5 and 22.3 ± 1.9 g, respectively; they did not significantly differ from each other, from Group 4, or from the negative control groups. Similarly, there were no differences in average food consumption among the six groups (data not shown). Six mice from Group 4, four mice from Group 5 and one mouse from Group 6 were sacrificed or died before the end of the experiment due to severe diarrhea and rectal bleeding and were excluded from the results of the carcinogenesis experiment. Histological examination of the colons from these mice showed extensive ulceration and edema (toxic megacolon). There was no statistically significant increase in the number of early dead mice among the groups.

Inhibition of UC-associated carcinoma development by inositol and hexaphosphate inositol
No colorectal tumors were found in negative control mice in Groups 1, 2 and 3. The results on colorectal carcinoma development for Groups 4, 5 and 6 are shown in Table I. A total of 24 out of 34 (70.6%) mice in Group 4 harbored colorectal carcinomas. The average number of colorectal tumors per mouse (tumor multiplicity) for Group 4 was 1.26 ± 1.05 (mean ± SD). The average tumor volume for this group was 21.4 ± 5.2 mm³, with a range of 5.2–133.4 mm³. Significant reduction of tumor incidence was observed in inositol-treated mice in Group 5 (9/21, 42.9%; P < 0.05). A marked reduction of tumor incidence in IP6-treated mice was observed in Group 6 (12/24, 50.0%), but did not reach statistical significance (P > 0.05). The tumor multiplicity for Group 5 was 0.5 ± 0.7 tumors per mouse and the average tumor volume was 4.2 ± 1.9 mm³ (range: 0.52–33.5 mm³) and both parameters presented were statistically significant from Group 4 mice (P < 0.05). In Group 6, the tumor multiplicity was 0.8 ± 0.9 and the average tumor volume was 12.3 ± 4.1 (range: 4.7–65.4 mm³). There was a statistically significant difference in tumor volume (P < 0.05), but not tumor multiplicity from Group 4. All of the colorectal tumors were confirmed in serial H & E stained slides by light microscopy and classified as well-differentiated adenocarcinomas or mucinous adenocarcinoma as shown in Figure 1A–C. Among the groups, there was no difference in histopathological types of adenocarcinomas, and the majority of the tumors were mucinous adenocarcinomas.

Inhibition of UC inflammation grading by inositol compounds
Chronic active or inactive colitis was frequently observed in non-tumor colorectal mucosa and exhibited as ulceration, reactive hyperplastic epithelial change and glandular distortion and basal lymphoplasmacytosis, as shown in Figure 2A and B. Colorectal inflammation was graded histologically in

![Fig. 1](image-url) Representative morphology of UC-induced carcinoma. (A) Polypoid, well differentiated tubular adenocarcinoma, 125X; (B) well differentiated mucinous adenocarcinoma with invasion into muscularis propria, 125X and (C) enlargement using high magnification of the highlighted site in (B), 500X.
non-cancerous mucosa based on ulceration, glandular hyperplasia and area of inflammatory involvement, and expressed as a UC index, as described previously (50,54). The colons of Groups 1, 2 and 3 negative or compound control mice were morphologically normal. The results of the UC index in Groups 4, 5 and 6 are summarized in Table II. In all DSS-treated groups, inflammation was mild in the proximal colon, where increased mucosal thickness was observed, but active inflammation (ulceration/erosion) was rare or smaller. UC was moderately severe in the middle and distal colon, with extensive areas of inflammation, erosion/ulceration and cryptitis. Hyperplasia (characterized by thickening mucosa with elongated, branched and crowded glandular epithelia with decreased number of goblet cell, and columnar cells often containing hyperchromatic nuclei) was commonly observed in these regions. The UC indices, ulcer formation and inflammation involved area in Group 5 did differ significantly from those of Group 4 in the overall colon. Marked inhibition of the UC index and inflammatory parameters in the colon were found in mice treated with IP6 in Group 6; but was not statistically significant in comparison to Group 4.

Table II. Inhibition of colorectal inflammation by inositol and hexaphosphate inositol

<table>
<thead>
<tr>
<th>Groups</th>
<th>Histological score</th>
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<tr>
<td></td>
<td>Severity Ulceration Hyperplasia Area involved UC index</td>
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<tr>
<td>Group 4</td>
<td>1.64 ± 0.03</td>
<td>0.61 ± 0.10</td>
<td>1.70 ± 0.16</td>
<td>3.28 ± 0.13</td>
<td>7.22 ± 0.30</td>
</tr>
<tr>
<td>Group 5</td>
<td>1.48 ± 0.06</td>
<td>0.36 ± 0.17</td>
<td>1.23 ± 0.14</td>
<td>1.38 ± 0.06</td>
<td>4.54 ± 0.13</td>
</tr>
<tr>
<td>Group 6</td>
<td>1.50 ± 0.06</td>
<td>0.55 ± 0.17</td>
<td>1.40 ± 0.14</td>
<td>2.48 ± 0.06</td>
<td>5.93 ± 0.13</td>
</tr>
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</table>

Data are expressed as the mean ± SE. The mice were subjected to 15 cycles of DSS treatment as described in Materials and methods. UC was scored in non-cancerous mucosa of the colon. Three H & E-stained slides per mouse from 6 mice per group were analyzed. The histological scores for each animal were the average of the scores for the three slides. The UC index was the sum of the individual scores for disease severity, ulceration, glandular hyperplasia, and area of inflammatory involvement. The criteria used for the histological scoring of UC are described in Materials and methods. *P ≤ 0.05 (Student’s t-test).

Mucosal Mac3-positive cell infiltration and nitrotyrosin formation

Anti-Mac3 antibody-labeled active macrophages were analyzed immunohistochemically in order to test the effect of inositol compounds on macrophage-mediated inflammation. Intensely stained Mac3-positive macrophages occasionally presented in the lamina propria in the colon of normal control mice and were frequently observed in the non-inflamed area of the colon in UC mice (Figure 3A). A decreased number of Mac3-positive cells were observed in non-inflamed areas of the colon from 6 mice in Groups 5 and 6 (Figure 3B and C). As shown in Table III, Mac-positive cell numbers (the mean number of Mac-positive cells per area of mucosa) in non-flamed colonic mucosa in UC mice presented a statistically significant difference between Group 4 and Group 5 (541 ± 59 versus 302 ± 65 Mac3-positive cells/mm² mucosa). However, no significant difference was observed between Group 4 and Group 6 (541 ± 59 versus 419 ± 48 Mac3-positive cells/mm² mucosa). It was shown that the administration of inositol or IP6 markedly reduced Mac3-staining intensity and the number of Mac3-positive cells in the inflamed (ulcerated/erosion) mucosa of the colon as compared to that in Group 4 mice (Figure 3D–F). However, this staining was difficult to quantify due to the intensity of the staining and the different sizes of the lesions.

Nitrotyrosine accumulation was analyzed immunohistochemically to determine the effect of inositol compounds on inflammation-caused nitrosative damage. Nitrotyrosine immunostaining showed strongly positively in the macrophages in non-inflamed and inflamed mucosa of the colon and in adjacent epithelia in UC mice, as shown in Figure 4A and C. Inositol and IP6 markedly reduced nitrotyrosine staining intensity and the number of nitrotyrosine positive cells in both non-inflamed and inflamed areas of the colon, as shown in Figure 4B and C, and 4E and F. As shown in Table III, nitrotyrosine-positive cell numbers (the mean number of positive cells per area of mucosa) in non-flamed colonic mucosa in UC mice were statistically significantly
different between Group 4 and Group 5 (638 ± 87 versus 389 ± 71 positive cells/mm² mucosa). However, no significant difference was observed between Group 4 and Group 6 (638 ± 87 versus 501 ± 67 positive cells/mm² mucosa). Similarly as seen in Mac-3 immunostaining, it was shown that administration of inositol or IP6 markedly reduced nitrotyrosine-staining intensity and the number of positive cells in the inflamed (ulcerated/erosion) mucosa of the colon as compared to Group 4 mice (Figure 4D–F). Again, this staining was difficult to quantify due to the intensity of the staining and the different sizes of the lesions.

Colonic epithelial and tumor cell proliferation

Proliferating epithelial cells were visualized by PCNA immunostaining. The PCNA immunostaining index was quantified in non-inflamed epithelia, hyperplastic/reactive epithelia and carcinomas. In the normal colons from the negative control or compound control mice, PCNA-positive cells occupied the lower third (base) of the colonic glands (the crypt region), as shown in Figure 5A. The colons of Group 4 mice showed an expansion of the crypt region and an increase in PCNA-positive cell numbers in morphologically normal mucosa and glandular distorted mucosa (Figure 5B and C). Hyperplastic/reactive epithelia in areas of active inflammation showed higher PCNA labeling indices as compared to non-inflamed epithelia. Both mucinous and well-differentiated tubular adenocarcinomas exhibited high PCNA-labeling indices immunohistochemically (Figure 5D and F). There was a marked difference between DSS-treated mice and DSS plus inositol or hexaphosphate inositol treated mice in the proliferation index in both non-inflamed and inflamed epithelia of the colon, as summarized in Table IV. Statistically significant inhibition of cell proliferation in non-inflamed or inflamed epithelia was observed in mice treated with inositol (P < 0.05); but not in mice treated with IP6. No significant difference was observed in the proliferation index of adenocarcinoma in mice treated with DSS as compared with the mice treated with DSS plus inositol or IP6.

Discussion

Several studies indicate that inositol and IP6 have strong anti-carcinogenic activity in chemical carcinogen-induced carcinogenesis in either mice or rats. The present study first demonstrated that inositol and IP6 significantly inhibit inflammation-driven carcinogenesis in a novel DSS-induced and iron-enhanced UC associated carcinoma model in mice without the use of a carcinogen. Based on our experiment design, inositol or IP6 is administered during the non-DSS period in the cyclic administration of DSS. Such design may result in lower inhibitory effects on UC-associated carcinogenesis than continuous administration; however, it allows us

![Fig. 3. MAC-3 labeled macrophages(brown) immunohistochemically and counterstained with hematoxylin for nuclei (blue). (A–C) Non-inflamed mucosa showing MAC-3 labeled macrophages in lamina propria (A) DSS only, (B) DSS and 1% IP6, (C) DSS and 1% inositol); (D–F) Abundant MAC-2 labeled macrophages in inflamed areas of the mucosa (D) DSS only, (E) DSS and 1% IP6, (F) DSS and 1% inositol).](image-url)
Fig. 4. Nitrotyrosine formation (brown) as detected immunohistochemically and counterstained with hematoxylin for nuclei (blue). (A–C) Non-inflamed mucosa showing nitrotyrosine formation mainly in the macrophages of the lamina propria (A): DSS only, (B): DSS and 1% IP6, (C): DSS and 1% inositol); (D–F) Intense nitrotyrosine-staining in inflamed areas of the mucosa, mainly in macrophages (D): DSS only, (E): DSS and 1% IP6, (F): DSS and 1% inositol).

Fig. 5. PCNA-labeled cell proliferation (brown) immunohistochemically and counterstained with hematoxylin for nuclei (blue). (A) Normal mucosa in negative control mice showing a few PCNA-labeled proliferative cells in the crypts of the colon; (B and C) Non-inflamed areas of colonic mucosa in DSS-induced chronic colitis showing markedly increased PCNA-labeled proliferative cells in cryptal areas of the colon (B) mucosa without glandular distortion, (C) mucosa with glandular distortion), (D and E) PCNA-labeled cell proliferation in mucinous adenocarcinoma (E) is an enlargement of the marked site in (D); (F) PCNA-labeled cell proliferation in polypoid adenocarcinoma.
to avoid any possibility of the direct or indirect interaction between DSS and inositol compounds and to generate more accurate, reliable and significant results. Interestingly, in comparison to inositol, the effect of IP6 on the inhibition of UC-associated carcinogenesis (tumor multiplicity and tumor incidence) is much smaller and does not have statistical significance. Similar results have been found in chemical carcinogen-induced tumors in mice, but not in rats (55–58), suggesting that it is possibly due to the difference in absorption and metabolism of inositol polyphosphate compounds in different species of animals. Further dose dependent studies are needed to confirm such possibilities or other mechanisms which may be involved.

Iron is an important risk factor to enhance UC-associated carcinogenesis (42,44,50). In our UC-carcinogenesis model, iron supplementation significantly enhanced carcinoma development (50). One possible mechanism of dietary iron enhancing UC-associated carcinogenesis is that dietary iron remains largely unabsorbed in the gastrointestinal tract, which interacts with reactive oxygen species to enhance oxidative stress caused DNA damage and carcinogenesis. The metal-chelating property of IP6, a highly charged compound, is one of its anti-oxidative stress functions and could also be counted as one of the mechanisms of its anti-cancer activities (59,60). Obviously, this function should be considered first in its anti-UC-associated carcinogenesis in the present study. The facts of several experimental evidences indicate that this chelating function is not a key event in the inhibition of carcinogenesis (61). Iron deficiency is common in this model as detected histochemically for iron storage in the spleen and liver in our previous study (50,62). Theoretically, if dietary iron binds to IP6, it will significantly affect iron nutrition status, and will result in more severe iron-deficiency anemia, loss of body weight and reduction of tumor development. Besides aforementioned tumor incidence and multiplicity, our results showed that administration of IP6 in both DSS and non-DSS treated mice exhibited similar iron nutrition status (iron storage and anemia) and indistinguishable body weights. Inositol, as a non-phosphorylated form, shows more potent anti-cancer activity than that by IP6 in the present study, indicating that chelating iron by IP6 is not a mechanism to reduce the dietary iron effect on UC-associated carcinogenesis. In addition, chemically, inositol does not contain any charged group to bind iron or metals and exhibits a stronger effect on inhibiting carcinogenesis, indicating other mechanisms are involved. Pharmacokinetically, administration of IP6 to either mice or rats shows quick dephosphorylation and absorption as a lower phosphorylated form or as inositol, suggesting that the lower phosphorylated form of inositol compounds may participate in the modulation of inositol signaling and cellular function on the inhibition of UC-associated carcinogenesis.

Interaction between inflammatory cells (prominent neutrophils in active inflammation and prominent macrophages, lymphocytes and plasma cells in chronic inflammation) and epithelial cells either through nitric oxide (NO) and reactive oxygen species or through cytokins and inflammatory mediators, such as prostaglandins play crucial roles in the initiation, promotion and progression of carcinogenesis (42). The inducible isof orm of nitric oxide synthase (iNOS), originally identified in macrophages, exhibits calcium-independent functions and is induced by pro-inflammatory cytokines. The activation of iNOS leads to prolonged production of NO in high, potentially cytotoxic concentrations (63). Tissues exposed to high concentrations of NO over long periods of time, such as in long-standing UC, could develop genetic instability and accumulate mutations either through the direct action of NO or through the interaction of NO with superoxide to form highly reactive peroxynitrite (49). Nitrotyrosine, a biomarker of NO-mediated protein modification, is commonly used to detect NO-mediated cellular damage. In the present study, marked reduction of nitrotyrosine formation, as detected immunohistochemically for its staining intensity and positive cell number, is observed in mice treated with inositol and IP6, strongly suggesting that its anti-inflammation action may be through inhibition of nitrosative and oxidative stress. A parallel immunohistochemical study of macrophages revealed that the number of macrophages in the lamina propria of the colon is reduced by inositol and IP6 treatment. Taken together, anti-inflammation action by inositol compounds may be through the inhibition of macrophage mediated inflammation.

Various phosphorylated or non-phosphorylated forms of inositol compounds exist in mammalian cells, where they participate in a variety of important cellular functions (1–6). Inositol phosphate metabolism is a known form of intracellular signaling, primarily in the cytoplasm. Recent studies indicate that inositol phosphates may also be involved in nuclear processes, including mRNA transport, transcriptional regulation and DNA repair (8–11). Taken together, modulation of cell signaling by inositol compounds may play a crucial role in the inhibition of carcinogenesis. These signals may associate with the regulation of cell proliferation, differentiated, apoptosis, DNA repair and immunity function. Hyperproliferation under long-term chronic inflammation status is a critical condition in mutagenesis and carcinogenesis. In the present study, significant inhibition of proliferation in hyperplastic epithelial cells of non-inflamed and inflamed mucosa by inositol compounds would be the most important aspect of anti-cancer activity, particularly on slowing down the progression of carcinogenesis. Several mechanisms should be considered for this anti-proliferation activity, such as the regulation of cell signaling. Further studies in animal models or in cell systems are needed to reveal its mechanism. Interestingly, inositol compounds exhibit a different effect on non-cancerous epithelial cells and carcinoma cells in the present study. Inositol-treated mice exhibit smaller tumors; but the rate of cell proliferation is not significantly inhibited as that observed in non-cancerous epithelial cells. Although

### Table IV. Inhibition of cell proliferation by inositol compounds

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<thead>
<tr>
<th>Groups</th>
<th>Non-inflamed mucosa %</th>
<th>Inflamed mucosa %</th>
<th>Cancer %</th>
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<tr>
<td>Group 4</td>
<td>54 ± 5.9</td>
<td>57 ± 6.7</td>
<td>67 ± 6.5</td>
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<tr>
<td>Group 5</td>
<td>36 ± 4.7</td>
<td>40 ± 4.8</td>
<td>55 ± 5.7</td>
</tr>
<tr>
<td>Group 6</td>
<td>45 ± 5.1</td>
<td>49 ± 5.6</td>
<td>58 ± 6.6</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SE. The mice were subjected to 15 cycles of DSS treatment as described in Materials and methods. Proliferation index was scored in non-inflammed, inflammed regions of non-cancerous mucosa of the colon and cancer regions. The proliferative PCNA-labeled cells were counted in more than ten areas of each lesion in total more than 2000 cells. The percentage of PCNA-labeled cells expressed as proliferation index.

*P ≤ 0.05 (Student’s t-test).
the mechanism related to this disparity is unknown, it is possibly due to the difference in cell proliferation control mechanisms between non-cancerous epithelia and carcinoma.

UC and its associated carcinoma development are a complex process (42,64,65). Inhibition of cell proliferation is important for the prevention of carcinoma development (42,65). However, this activity may delay the mucosa ulcer healing and exacerbate inflammation. For example, such effects have been observed in UC patients with the use of non-steroid anti-inflammatory drugs, such as Aspirin. Furthermore, inhibition of both cell proliferation and inflammatory activity is crucial for the development of an efficient strategy to prevent UC-associated carcinogenesis. Inositol is one of such agents that exhibit the effect on the inhibition of UC-associated carcinogenesis and on the suppression of inflammatory cells, reduction of UC index and ulcer size, and inhibition of cell proliferation.

In conclusion, inositol compounds showed significant inhibition of UC-associated carcinoma development in mice, in association with suppression of cell proliferation and macrophage-mediated inflammation and nitro-oxidative stress. The present findings strongly suggest that inositol compound could be a strong candidate agent in the prevention of carcinoma development in UC patients, especially in patients with such a high risk (i.e. the patients with UC of more than 10 years or pancolitis).

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References

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