Prevention of Colitis-Associated Colorectal Cancer with 8-Hydroxydeoxyguanosine

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Abstract

Colitis-associated cancer (CAC) is one of clear examples of inflammation–carcinogenesis sequence, by which the strict control of colitis with potent anti-inflammatory or antioxidative agent offers the chance of cancer prevention. Supported with the facts that Rac1 binds and activates STAT3, which are significantly upregulated in inflammatory bowel disease (IBD) as well as CAC, but 8-hydroxydeoxyguanosine (8-oxo-7,8-dihydrodeoxyguanosine or 8-OHdG) paradoxically can block Rac1 activation and subsequent NADPH oxidase (NOX) inactivation in various inflammation models, we hypothesized that attenuated Rac1–STAT3 and COX–NF-κB pathway by exogenous 8-OHdG administration may ameliorate inflammatory signaling in dextran sodium sulfate (DSS)-induced colitis and can prevent CAC. Before commencing carcinogenesis model, we checked whether exogenous 8-OHdG can alleviate IBD, for which interleukin (IL)-10 knockout mice were designed to ingest 5% DSS for 1 week, and 8-OHdG is given through intraperitoneal route daily. 8-OHdG treatment groups significantly reduced pathologic grade of DSS-induced colitis as well as various inflammatory mediators such as TNF-α, IL-6, COX-2, and iNOS in a dose-dependent manner. To document the cancer prevention effects of 8-OHdG, mice were injected azoxymethane followed by drinking 2.5% DSS for 1 week, after which 8-OHdG–containing diets were given for 20 weeks. As results, mice that consumed 8-OHdG–containing diet significantly reduced both tumor incidence and multiplicity. Rac1 activity and phosphorylated STAT3 level were significantly attenuated in the 8-OHdG–treated group. Significantly decreased levels of malondialdehyde, monocyte chemotactic protein-1, matrix metalloproteinasess, COX-2, NOX4, and β-catenin nuclear accumulation were responsible for cancer prevention effects of exogenous 8-OHdG. In conclusion, we clearly showed cancer-preventive effect of exogenous 8-OHdG against CAC. Cancer Prev Res; 4(9): 1507–21. ©2011 AACR.

Introduction

Ulcerative colitis and Crohn’s disease are a form of chronic inflammatory bowel disease (IBD) that usually takes a clinical course of repeated bouts of inflammation and remission. IBD can not only cause problematic sequelae that needed extensive surgical procedures but also increases risk for the development of colitis-associated colorectal cancer (CAC), also known as colitic cancer (1, 2). In contrast to “adenoma–carcinoma sequence” of sporadic colorectal cancer (CRC), CAC is one of well-known examples of “inflammation–dysplasia–carcinoma sequence” that reversely implies that modulating colon inflammation by efficacious anti-inflammatory treatment at early phase of disease may more likely inhibit subsequent carcinogenesis (3, 4). However, it seems to be very difficult to control the activity of disease continuously with each or combination of anti-inflammatory agents such as 5-aminosalicylic acid or other immunosuppressive drugs such as glucocorticoids and 6-mercaptopurine (5). Although biologics have been reported to be effective in either induction of remission or treatment of complications as well as the preventive strategy of colitic cancer (4), there remains room for improvement and newer agent should be investigated.

Pathogenesis of CAC includes complex factors of inflammation-initiated or -promoted mutagenesis process. That is, mucosal inflammation is provoked by releasing chemokines to recruit and activate inflammatory cells (6, 7) and turning the arachidonic acid cascade on mediation by upregulation of COX-2 and downregulation of 15-hydroprostaglandin...
dehydrogenase (PGDH; refs. 8, 9). Nuclear factor kappaB (NF-κB) is known to play a main role in these early steps of inflammation (10, 11), but it can also provoke other inflammatory cascades such as IL-6/JAK/STAT3 signaling pathways to destroy adjacent tissues by activating matrix metalloproteinase (MMP) complexes (12, 13) and to turn

Figure 1. Efficacy of exogenous 8-OHdG on DSS-induced acute colitis. A, overview of experimental protocol of DSS-induced colitis. Top, 5 groups, normal control, 5% DSS-induced colitis group (DSS), 50 mg/kg, 100 mg/kg, and 150 mg/kg 8-OHdG i.p. injection group. Bottom, the changes of body weight, colon length, and incidence of hematochezia according to groups. Bar represents mean ± SD. B, the changes of pathologic index according to groups. Bar represents mean ± SD.

* P < 0.05
on mutagenesis process even in normal crypts (14, 15). When mutagenesis occurs in intestinal epithelium at the very first time, APC–β-catenin pathway is disturbed by loss of APC function that normally degrades β-catenin, which eventually causes cell proliferation and confers resistance to apoptosis. Increased levels of β-catenin may translocate into nucleus and act as transcription factor with Tcf/Lef complex, turning normal gland into dysplastic gland (16–18). Following these mutagenesis sequences, mutations of K-Ras and p53 as well as microsatellite instability pathway, caused loss of heterozygosity and progressed more up to aggressive nature.

Figure 1. (Continued) C, Western blot of COX-2, iNOS, and HO-1 according to group. Three representative cases of 6 mice per group were presented. Bottom, the expression levels of the ratio of COX-2 to β-actin, iNOS to β-actin, and HO-1 to β-actin were measured by densitometry. Bar represents mean ± SD. D, ELISA levels of mucosal TNF-α and IL-6 according to groups. Bar represents mean ± SD.

Prevention of Colitic Cancer with 8-OHdG
Although 8-hydroxydeoxyguanosine (8-oxo-7,8-dihydrodeoxyguanosine or 8-OHdG) has been acknowledged as a sensitive marker for oxidative stress, a potent mutagen or a toxic product, we previously published that 8-OHdG specifically inhibits Rac1/2, the members of small GTPase family that regulate the genes associated with inflammation–dysplasia–carcinoma sequence.

All of these backgrounds led us to investigate the hypothesis that exogenous administration of 8-OHdG might have the preventive effect on the animal model of colitic cancer based on its potent anti-inflammatory and antioxidative actions (19–22). Using dextran sodium sulfate (DSS)-induced colitis and 2-stage colon cancer model first established by Tanaka and colleagues (23), in which colon cancer is initiated by azoxymethane (AOM) and promoted by DSS ingestion, we studied whether exogenous 8-OHdG is effective in ameliorating colitis, after which long-term administration of 8-OHdG–containing diet can prevent colitis-associated cancer in interleukin (IL)-10 knockout mice to prove the potential role of exogenous 8-OHdG as a natural terminator or means of negative feedback of inflammation in chronic inflammation–carcinogenesis sequence.

Materials and Methods

Materials
All chemicals and reagents were purchased from Sigma-Aldrich otherwise specified. Primary antibodies for Western blotting and immunohistochemistry were purchased as follows: NOX4, β-actin, β-catenin, and STAT3 from Santa Cruz Biotechnology, iNOS from BD Biosciences, HO-1 from Enzo Life Sciences, COX-2 from Thermo Scientific, 15-PGDH from Cayman Chemical, phosphorylated STAT3/S727 from Cell Signaling Technology, and F4/80 from AbD Serotec.

Breeding of IL-10 knockout mice
Two pairs of breeding IL-10 knockout mice (−/−) and genotyping primers were purchased from Jackson Laboratory. Background of IL-10−/− mice was C57BL/6J. Female and male IL-10−/− mice were mated, and all offspring of mice were considered as homozygote after genotyping. A total 70 of 5-week-old specific pathogen-free (SPF) IL-10−/− mice were fed a sterilized commercial pellet diet (DH Biolink), given sterile water ad libitum, and housed in an air-conditioned room under a 12-hour light/dark cycle.

Animals were handled in an accredited animal facility in accordance with Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) guidelines under the facility named CACU (The Center of Animal Care and Use) of Gachon University Lee Gil Ya Cancer and Diabetes Institute after Institutional Review Board approval.

Animal model of DSS-induced colitis
A total of 30 mice were divided into 5 groups, 6 mice per group, respectively; a nontreated control group (normal), 5% DSS (molecular weight = 36,000–50,000; MP Biomedicals) in tap water ingestion for 1 week (DSS), and DSS with daily intraperitoneal (i.p.) injection of 8-OHdG 50, 100, and 150 mg/kg groups. Powers of 8-OHdG was dissolved in PBS and mice of the normal group and the DSS group were injected with PBS i.p. as a negative control. Clinical phenotypes such as hematochezia and rectal prolapse were investigated and charted daily. There were no mortalities observed in all groups. After 7 days of DSS ingestion, all mice were killed and colons were removed, opened longitudinally, and rinsed with PBS. The lengths of colon were measured, and isolated tissues were subjected to a histologic examination and extraction of protein.

Animal model of DSS-induced colitis and 2-stage colitic cancer
The remaining 40 mice were divided into 4 groups, 10 mice per group, respectively; a nontreated control group (normal), AOM 10 mg/kg injection followed by 1 cycle of 2.5% DSS ingestion for 1 week after which mice were fed with normal AIN-76 diet group (AOM + DSS), and AIN-76–containing 8-OHdG 0.03% diet (8-OHdG 0.03%) or 8-OHdG 0.06% diet group (8-OHdG 0.06%). Mice of the normal group were injected with PBS i.p. and drank tap water. A total of 4 mice were dead during 2.5% DSS ingestion: 1 in the AOM + DSS group, 2 in the 8-OHdG 0.03% group, and 1 in the 8-OHdG 0.06% group, respectively. Daily consumption of 8-OHdG per animal was estimated approximately 50 mg/kg/d for the 8-OHdG 0.03% group, and 100 mg/kg/day for the 8-OHdG 0.06% group, respectively. After 20 weeks of diet, all mice were killed and colons were removed, opened longitudinally, and rinsed with PBS. The number and size of gross polyoid tumors were charted. Isolated tissues from distal one-third of colon were subjected to a histologic examination and mucosal scratches of tissues from proximal two-third part were subjected to immediate freezing for the extraction of RNA and protein.

Histopathologic evaluation
Isolated tissues for histologic examination were spread onto a plastic sheet, fixed in 3.7% formalin for 5 hours, and prepared for paraffin tissue slides (25). The paraffin sections were stained with hematoxylin and eosin (H&E) or saved for immunohistochemical staining. Pathologic index was graded according to criteria as shown in Supplementary
Table S1. Colitis-associated colon neoplasms were analyzed microscopically and diagnosed as low-grade dysplasia, high-grade dysplasia, and adenocarcinoma. Tumor incidence was calculated as the number of tumor-bearing mice divided by the total number of survival mice, whereas tumor number was calculated as the total number of tumors divided by the number of tumor-bearing mice. Tumor size was calculated as the sum of size of tumors divided by the...
number of tumor-bearing mice, and percentage of mucosal surface with tumor was grossly estimated. Pathologic data and slides were blindly reviewed by 2 independent gastrointestinal specialist (K.B. Hahm and Y.J. Kim) and the first author (C.Y. Ock).

**Cytokine array and ELISA**

Raybio mouse cytokine antibody array 3 were purchased from RayBiotech and carried out strictly according to manufacturer’s instruction. Three hundred micrograms of a representative case of each group was used for this assay. ELISA kits for mouse TNF-α, mouse IL-6 (R&D Systems), mouse monocyte chemotactic protein-1 (MCP-1; Invitrogen), and prostaglandin E2 (PGE2; Cayman) were purchased and used strictly according to the manufacturer’s instruction.

**Reverse transcriptase PCR, Western blots, electrophoretic mobility gel shift assay**

This assay was carried out as previously described (19). The sequences of primers are listed in Supplementary Table S2.

**Rac1 activity assay**

This assay was carried out as previously described (19). Rac1 activity was analyzed by determining the amount of GTP-bound Rac1 pulled down by interacting GST–PAK–PBD.
(p21 activated kinase–protein binding domain) with Rac1 using the Rac1 activation assay (cytoskeleton), according to the manufacturer’s instructions. Four hundred micrograms of a representative case of each group was used for this assay.

**Immunohistochemistry and immunohistofluorescence**

Immunohistochemistry with anti-F4/80 antibody and anti-β-catenin antibody was carried out as previously
described (4). For immunohistofluorescence study, the sections were deparaffinized and blocked with 2.5% goat serum for 30 minutes, incubated for 12 hours at 4°C with antibody against NOX4 (1:100 dilution), and finally incubated for 1 hour with Alexa Fluor 488 goat anti-rabbit immunoglobulin G (Invitrogen) in the presence of 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). The slides were mounted with Prolong Gold anti-fade agent (Invitrogen) and inspected using a confocal microscope (LSM700; Carl Zeiss).

**Total malondialdehyde assay**

MDA-586 kit was purchased from Oxis International and used according to the manufacturer’s instructions. To measure total level of malondialdehyde (MDA), isolated tissues were incubated with PBS (pH 2.0) for 80 minutes at 60°C before carrying out this assay.

**Statistical analysis**

The data are presented as means ± SD. The Tukey test or the Student t for unpaired results was used to evaluate differences between more than 3 groups or between 2 groups, respectively. Differences were considered to be significant for values of \( P < 0.05 \).

**Result**

**The ameliorating efficacy of exogenous 8-OHdG on DSS-induced colitis**

We used IL-10 KO mice, IBD-prone mice (26), to provoke intense colitis in the SPF environment of our animal facility. As shown in Figure 1A, 5% DSS administration provoked significant levels of colitis as reflected with gross findings that the body weight of mice and the length of colon were significantly decreased accompanied with significantly
increased anal bleedings. However, i.p. injection of 8-OHdG leads to increases in the length of colon as well as maintenance of body weight in spite of DSS administration ($P < 0.05$). On pathologic analysis, DSS administration distorted glandular formation and recruited inflammatory cells especially in submucosal layer, leading to mucosal destruction, whereas 8-OHdG treatment significantly attenuated these pathologic indices as shown in Figure 1B. In addition, the inflammatory mediators such as COX-2 and iNOS were increased in 5% DSS-induced colitis compared with normal control but significantly decreased in the 8-OHdG injected group in a dose-dependent manner. The expression level of HO-1 was increased in the DSS group, speculated to be a part of host defense mechanism, but they were further increased with 8-OHdG treatment (Fig. 1C). The levels of mucosal TNF-α and IL-6, crucial cytokines engaged in colitis initiation and propagation, were far increased in the DSS-administered group, but their levels were significantly decreased with 8-OHdG treatment (Fig. 1D).

**The preventive effect of 8-OHdG–containing diet on colitic cancer induced by AOM + DSS**

IL-$10^{-7}$ mice injected with AOM followed by 2.5% DSS in drinking water for 1 week developed colorectal tumors at 20th week after DSS ingestion (Fig. 2A). Most tumors were concentrated in distal one-third part where we found a variety of stages of cancer progression such as dysplasia, adenoma, and adenocarcinoma in histologic examination (Fig. 2B). Mice fed with 8-OHdG–containing diet 0.03% or 0.06% decreased either incidence or size of tumor especially in proximal two-third part of colon (Fig. 2C). The exact numbers of incidence, number, and size of tumors as well as percentage of mucosal surface with tumor were summarized in Supplementary Table S3. In addition to these antitumorigenic outcomes of 8-OHdG evaluated with the numbers and sizes of colitic tumor, carcinogenesis progress was trapped in adenoma in the 8-OHdG–treated group compared with high incidence of adenocarcinoma in the AOM + DSS group.

**Attenuated footprint of inflammation on colitic cancer administered with 8-OHdG–containing diet**

To understand what kinds of cytokine were increased in colitic tumors of the AOM + DSS group compared with normal mice, we carried out mouse cytokine antibody array. As shown in Figure 3A, abundant cytokines such as series of IL, TNF, as well as chemokines such as MCP-1, P-selectin, and RANTES were increased in the representative case of the AOM + DSS group, of which levels were decreased in the representative cases of the 8-OHdG 0.03% and 8-OHdG 0.06% groups, respectively (Fig. 3A). Because these cytokines and chemokines were essential in chemotaxis including macrophages, we carried out immunohistochemical staining with anti-F4/80 antibody, a well-known marker for macrophage. The number of cells expressing F4/80 was increased in the AOM + DSS group compared with the normal group, but chronic consumption of 8-OHdG–containing diet decreased the recruitment of F4/80-positive cells. Similar to cytokine array, MCP-1 levels upregulated in the AOM + DSS group were significantly decreased with 8-OHdG in a dose-dependent manner (Fig. 3B). On the basis of array results, we checked the expression of MMPs, well known to be engaged in invasion as well as metastasis of colitic cancer (13). Figure 3C showed that the mRNA levels of MMP-2, MMP-3, MMP-9, and MMP-11 were increased in the AOM + DSS group, but significantly decreased with 8-OHdG 0.03% and 8-OHdG 0.06% administration. The mRNA levels of other series of MMPs such as MMP-7, MMP-13, TIMP-1, TIMP-2, MT-MMP1, and MT-MMP2 were measured, but there were no significant changes between groups (data not shown).

**Attenuated prostaglandin pathway with 8-OHdG–containing diet**

Increased inflammatory foot prints as shown in Figure 3 were reflected with increased status of oxidative stress, leading to increased lipid peroxidation in colonic mucosa. Figure 4A showed the results of MDA to reflect lipid peroxidation. As anticipated, AOM + DSS induced significant levels of MDA formation, whereas their levels were significantly decreased with 8-OHdG administration. Because many have reported that increased expression and activity of COX-2 led to the activation of arachidonic pathway, mainly, PGE2 upregulation, in inflammatory bowel disease as well as colitic cancer, whereas decreased expression and activity of PGDH were found in CRC, we checked expression levels of COX-2 and PGDH according to groups. As expected, the significantly increased expressions of COX-2 and decrements in PGDH expressions were observed in the AOM + DSS group (Fig. 4B). The expression levels of COX-2 were significantly reduced in the 8-OHdG 0.03% and 8-OHdG 0.06% groups compared with the AOM + DSS group, but there were no changes in the expression levels of PGDH between those groups. To check the changes of COX-2 activity, we measured tissue levels of PGE2 using homogenates of isolated colon. PGE2 was significantly increased in the AOM + DSS group, of which levels were significantly attenuated in the 8-OHdG 0.03% and 8-OHdG 0.06% groups compared with the AOM + DSS group (Fig. 4C). As NF-kB signal pathway was intimately involved in upregulation of COX-2 (27), we checked the DNA binding activity of NF-kB, which was markedly elevated in the AOM + DSS group, but its DNA bindings were significantly decreased in the 8-OHdG 0.03% and 8-OHdG 0.06% groups, in a dose-dependent manner (Fig. 4D).

**Attenuated Rac1–STAT3 pathway with resultant NOX4 inhibition with 8-OHdG**

Previously, we have reported that the biological target of exogenously administered 8-OHdG would be Rac1, the small GTPase, as it also has guanine base that could interrupt GTP binding (19, 28). Although best evaluation might be to document 3-dimensional analysis of binding site as well as more sophisticated designs to confirm biological interaction between 8-OHdG and the target site, in the
current study, we hypothesized that if the main target of 8-OHdG for chemopreventive effect of the AOM + DSS model is to inhibit Rac1 binding, 8-OHdG could inactivate STAT3, a well-known significant transcription factor in initiation and progression of colitic cancer as well as sporadic CRC (29, 30). As expected, the activity of Rac1 was far increased in the AOM + DSS group, but interestingly, dramatically decreased in the 8-OHdG 0.03% and 8-OHdG 0.06% groups, in a dose-dependent manner (Fig. 5A). Because active form of Rac1 can directly bind to STAT3 (21, 23),

Figure 5. Changes of Rac1–STAT3 pathway according to groups. A, Rac1 activities of a representative case of 8 mice per group were assayed by immunoprecipitation (IP) using PAK–PBD beads that specifically precipitate with Rac1–GTP, the active form of Rac1. IB, immunoblotting. Activated Rac1 also bound with STAT3. The levels of Rac1 and STAT3 in whole lysate were presented as a loading control. B, Top, Western blots were probed with anti-phospho-STAT3 (p-STAT3) and anti-STAT3 antibodies. Four representative cases of 8 mice per group were presented. Bottom, the expression levels of the ratio of p-STAT3 to STAT3 were measured by densitometry. Bar represents mean ± SD. C, changes of NOXs according to groups. The mRNA levels of NOX1, NOX2, and NOX4 were assayed by reverse transcriptase PCR. glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Top, the expressions of NOXs of a representative case of 8 were presented. Bottom, the expression levels of the ratio of NOX4 to GAPDH were measured by densitometry. Bar represents mean ± SD. D, Top, Western blots were probed with anti-NOX4 and anti-β-actin antibodies. Four representative cases of 8 mice per group were presented. Bottom, the expression levels of the ratio of NOX4 to β-actin were measured by densitometry. Bar represents mean ± SD.
we carried out further immunoprecipitation assay, immunoprecipitated with PAK–PBD beads, which specifically binds to active form of Rac1, and then immunoblotted with α-STAT3 antibody. AOM + DSS induced significant activation of STAT3 as shown in Figure 5A. In this condition, significantly blocked interaction of Rac1–STAT3 was noted in 8-OHdG group, much weak expression of STAT3 was observed in precancerous lesions. Bottom, the densities of NOX4 expression (515 nm) and DAPI (460 nm) were measured by a densitometer. Three independent regions of each slide were observed, and 4 slides of 8 mice per group were used for this experiment.

Figure 5. (Continued) E, Top, immunohistofluorescence of NOX4 with DAPI staining was carried out and a representative case of the normal, AOM + DSS, and 8-OHdG 0.06% groups were presented with H&E staining (×50). The aberrantly increased expression of NOX4 was observed in the AOM + DSS group, especially inside adenocarcinoma lesions. In the 8-OHdG 0.06% group, much weak expression of NOX4 was observed in precancerous lesions. Bottom, the densities of NOX4 expression (515 nm) and DAPI (460 nm) were measured by a densitometer. Three independent regions of each slide were observed, and 4 slides of 8 mice per group were used for this experiment.

we carried out further immunoprecipitation assay, immunoprecipitated with PAK–PBD beads, which specifically binds to active form of Rac1, and then immunoblotted with α-STAT3 antibody. AOM + DSS induced significant activation of STAT3 as shown in Figure 5A. In this condition, significantly blocked interaction of Rac1–STAT3 was noted in 8-OHdG–treated group in a dose-dependent manner. Because the binding of Rac1–STAT3 might lead to STAT3 activation, we further checked phosphorylation status of STAT3. The ratio of phopho-STAT3 to STAT3 was increased in the AOM + DSS group but significantly blocked in the 8-OHdG 0.06% group (Fig. 5B). We also assayed the DNA-binding activity of STAT3, which was increased in the AOM + DSS group but decreased in the 8-OHdG 0.03% and 8-OHdG 0.06% groups as well as nonlabeled competitor group (data not shown). Therefore, the inhibition of Rac1 activity leading to blocking Rac1–STAT3 binding might be the main mechanism of action of 8-OHdG on chemoprevention of murine model of colitic cancer.

We previously reported that regulating Rac1 activity by 8-OHdG might affect not only production of ROS in an acute phase but also upregulation of other subsets of NOX to generate ROS (19). Moreover, several investigators published that STAT3 pathway directly upregulated transcription of NOX1 and NOX4 in vitro (31, 32). Therefore, we hypothesized that the level of NOX would be changed during carcinogenesis of animal model of colitic cancer induced by AOM + DSS. First, we checked the mRNA levels of various subsets of NOX. Figure 5C showed that the mRNA level of NOX1 was decreased in the AOM + DSS group compared with the normal group, but there were no changes in the mRNA levels of NOX2 between groups. NOX3 was not detected in all groups, although Raw264.7, mouse macrophage cells, clearly expresses NOX3 (data not shown). Interestingly, the mRNA levels of NOX4 were far
increased in the AOM + DSS group compared with the normal group, and 8-OHdG–containing diet reduced its expression significantly in a dose-dependent manner. The protein levels of NOX4 were expressed in a manner similar to that of mRNA levels (Fig. 5D). Moreover, NOX4 was highly expressed inside and around cancer tissues, but the normal group and the 8-OHdG 0.06% group had low profiles of NOX4 expression (Fig. 5E).

Attenuated formation of β-catenin–accumulated crypt with 8-OHdG–containing diet

Because there was no significant difference in apoptosis between group (see Supplementary Fig. S1) and β-catenin–accumulated crypt (BCAC) is the useful marker for dysplastic lesions or aberrant crypt foci (ACF; ref. 17), the expression of β-catenin was visualized with immunohistochemical staining. As shown in Figure 6A, the colonic crypts of the AOM + DSS group were intensively stained with β-catenin, more prominently in nucleus of aberrant colonocytes. Right, the number of BCACs of groups was counted. Bar represents mean ± SD. B, immunoblot of β-catenin. Top, Western blots were probed with anti-β-catenin and anti-β-actin antibodies. Four representative cases of 8 mice per group were presented. Bottom, the expression levels of the ratio of β-catenin to β-actin were measured by densitometry. Bar represents mean ± SD.

Discussion

Similar results as shown in our previous issues (4, 25) that the prevention of CAC with either infliximab, biologics composed of TNF-α antibody or omeprazole, one of
proton pump inhibitors capable of inhibiting TNF-α, we can add more evidence showing that the application of a potent antioxidative and anti-inflammatory agent, 8-OHdG in the current study, can be an effective strategy to prevent inflammation-based carcinogenesis. Although the clinical significance of 8-OHdG as biomarker for oxidative stress has been well studied and associated with inflammation, mutagenesis, and carcinogenesis because it is easily detected in sera or urine of patients by its membrane permeability (33–35) because 8-OHdG might incorporate into genomic DNA, causing transversion mutation (36, 37), our group has clearly reported that paradoxically exogenous 8-OHdG exerted anti-inflammatory and antioxidant action in various kinds of inflammation-based animal model as well as in vitro experiment (19–22). Because exogenous 8-OHdG is not salvaged into genomic DNA, as it is to be neither degraded into 8-oxo-7,8-dihydroguanine nor phosphorylated into 8-oxo-7,8-dihydroguanine triphosphate and it indeed never does seem to incorporate into nucleus compared with deoxyguanosine control (38, 39), 8-OHdG can be a novel drug candidate. The current study might be the first publication proving that long-term efficacy of exogenous 8-OHdG administration in the prevention of colitis-associated carcinogenesis and inflammatory bowel disease. Although acute inflammation that persists for short term mediates host defense against infections, chronic inflammation that lasts for longer periods can predispose the host to various chronic illnesses, especially cancer, confirming a cross-link between inflammation and cancer.

Therefore, the strategy to cover the control of both acute and chronic inflammation seems to be the more efficacious way for preventing the journey to inflammation-associated carcinogenesis, for which the following mechanisms in addition to relieving action against DSS-induced acute colitis should be incorporated as the cancer-preventing elixir of exogenous 8-OHdG: first, the regulation of transcription factors NF-κB and STAT3, 2 major pathways for inflammation-based carcinogenesis as well as inflammation; second, potent anti-inflammatory action exerted in the forefront of colitic cancer development; third, the regulation of the most gene products linked to survival, proliferation, and mutagenesis; fourth, relieving oxidative stress; and finally, strict control of Wnt signaling including β-catenin accumulation and nuclear translocation. Successfully, 8-OHdG fulfilled all of these qualifications for the prevention of CAC.

In detail, Rac1 activity itself is also implicated in cancer progression, such as cytoskeleton rearrangement, cell migration, cell growth, and apoptosis (40, 41). In this study, we focused on STAT3 activation, NOX induction, and subsequent ROS generation by active form of Rac1 in colitis-associated cancer model. The activity of Rac1 is known to be a crucial organizer of NOX complex that is responsible for producing ROS, all of which in turn lead to a variety of inflammatory cascades (42). Previously, we reported that increased Rac1 activity may directly organized NOX2, a housekeeping gene in phagocytes, generating ROS in a very acute phase of treatment with LPS, but prolonged maintained ROS increased the expressions of other subsets of NOX, such as NOX1 and NOX4, in a murine macrophage cell line (19). Although the close correlation of Rac1 with NOX1 and NOX2 has been clearly clarified, the relationship of Rac1 with other subsets of NOX has not yet been proven. Recent report (43) showed that the expression levels of NOX1 and NOX4 were increased in human sample of colon cancers as well as animal model of carcinogen-induced colon cancers. In this current study, the expression level of NOX4 was dramatically increased but the mRNA level of NOX1 paradoxically seemed to be decreased by AOM treatment followed by DSS ingestion. Exogenous 8-OHdG significantly blocked NOX4 upregulation (Fig. 5). On the basis of a previous report by Manea and colleagues (31) that the transcription of NOX4 was increased directly by STAT3 pathway in smooth muscle cells and decreased Rac1 activity by 8-OHdG further mitigated STAT3 pathway by us (19), it might be possible that activated Rac1–STAT3 pathway directly induces the transcription of NOX4, generating more potent ROS, leading to activation of redox-sensitive transcription factor, NF-κB, and activating prostaglandin pathways (Fig. 4), all of which were known to be critically and principally involved in “inflammation–dysplasia–carcinoma sequence.” Conclusively, the cancer-preventive outcome of exogenous 8-OHdG also can be explained with blocking this “inflammation–dysplasia–carcinoma” sequence. Because the limited success of current treatments of most advanced common malignancies highlights the importance of cancer prevention, more efforts should be paid in cancer arising from inflammation basis as seen in our study.

Next focus relating to 8-OHdG and cancer prevention should be "COX." Between 2 forms of COX, COX-1 constitutively expressed in normal tissues and served as a "housekeeper" of mucosal integrity and COX-2 served as an immediate early response gene that is highly inducible by neoplastic and inflammatory stimuli. Increased levels of COX-2 mRNA and proteins are found in premalignant lesions and malignant tissues of colitic cancer, making it an attractive therapeutic target (44). Chemoprevention of CRC is already possible with celecoxib, although it is still not the ultimate drug of choice, especially because of the cardiovascular risk associated with COX-2 inhibitors, by which agent targeted for prevention of colon cancer should exert inhibition of COX-2 but maintenance of PGDH, tumor suppressor gene related to COX-2 (45, 46). Exogenous 8-OHdG was very efficient in either suppressing COX-2 expression or decreasing mucosal levels of PGE2 (Fig. 4B and C). Although the levels of PGDH were significantly decreased in AOM + DSS tumor-promoting condition compared with normal, clear preservation of PGDH was not seen in 8-OHdG treatment in spite of strong suppression of COX-2.

As much as colonocytes, cancer-associated fibroblasts (CAF) represent the predominant cell type of the neoplastic stroma of colon cancer, although their exact biology and...
functional specificity for cancer pathogenesis remain unclear. Mueller and colleagues (47) showed that primary CAFs from colorectal liver metastases express several inflammatory, tumor-enhancing factors, including IL-6 and MCP-1, both molecules intensely induced by TNF-α. Although not documented about detailed nature of expressing cell in this study, 8-OHdG imposed significant decreases in MCP-1 levels as well as inhibiting macrophage infiltration (Fig. 3A and B). In addition to MCP-1, several growth and angiogenic factors as well as matrix-degrading proteases, such as MMP-2, MMP-3, MMP-9, and MMP-11, are involved in general tumorigenesis, invasion, and metastasis as well as colon cancer. MMPs can be released in response to proinflammatory cytokines such as TNF-α and IL-1β and play an important role in the process of tissue remodeling and destruction, especially MMP-9 is the most abundantly expressed protease in inflamed tissues of colon. Also, MMPs, including MMP-2 and MMP-9, are expressed in human and animal cancers (48, 49). Exogenous 8-OHdG imposed significant inhibition of these tumorigenic actions of MMPs on colitic cancer. Finally, inactivating action of β-catenin accumulation was imposed with 8-OHdG. ACFs are now frequently used as effective surrogate biomarkers of CRCs, but the real preneoplastic or precancerous nature of ACFs in rodents and human still remains inconclusive (50). Instead of these obscuring implications of ACFs, early appearing BCACs have been described in en face preparations of colonic mucosa and are suggested to be premalignant in much higher fidelity than in ACFs. Histologic observation showed that BCACs exhibit cellular dysplasia, higher cellular proliferation, and more likely to progress to malignant transformation and this is why BCAC is acknowledged as an intermediate biomarker for colon carcinogenesis with higher fidelity (17). Therefore, significant decreases in BCAC with 8-OHdG as shown in Figure 6A might be one of the kernel evidence supporting cancer-preventing action of 8-OHdG. In addition, we found that there was no difference in apoptosis as evidenced by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining (see Supplementary Fig. S1).

In summary, the significance of our study is that we were successful in not only documenting the cancer-preventive effect of CAC with exogenous 8-OHdG, formerly recognized as the product of oxidative stress, but also elucidating the core pharmacologic actions of 8-OHdG, paradoxically antioxidative and anti-inflammatory (19–22) actions such as the regulation of Rac1–STAT3, NOX4, β-catenin, MMP, and COX-2, key mediators reported to be principally engaged in colon carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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