Intestinal Mucosal Inflammation Leads to Systemic Genotoxicity in Mice

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Abstract

Inflammatory bowel disease, including ulcerative colitis and Crohn’s disease, substantially increases the risk of colorectal cancer. However, mechanisms linking mucosal inflammation to the sequence of dysplasia are incompletely understood. Whereas studies have shown oxidative damage to the colon, this study tests whether genotoxicity is elicited systemically by acute and chronic intestinal inflammation. In this study, genotoxic endpoints were assessed in peripheral leukocytes (DNA single- and double-stranded breaks and oxidative DNA damage) and normochromatic erythrocytes (micronuclei) during chemical or immune-mediated colitis. During three consecutive cycles of intestinal inflammation induced by dextran sulfate sodium administration, genotoxicity to peripheral leukocytes and erythroblasts was detected in both acute and chronic phases of dextran sulfate sodium-induced inflammation. Reactive oxygen species–mediated oxidative stress and DNA damage was confirmed with positive 8-oxoguanine and nitrotyrosine staining in peripheral leukocytes. Levels of DNA damage generally decreased during remission and increased during treatment, correlating with clinical symptoms and systemic genotoxicity, such as in the peripheral blood. Accordingly, determination whether intestinal inflammation affects the level of systemic DNA damage in acute and chronic colitis has been confirmed by interventional studies (12–15). However, the mechanisms linking metabolic stress, inflammation, and cancer remain incompletely understood.

In addition to genetic models of chronic colitis, an important experimental model of colitis and colitis-associated neoplasia is administration of dextran sulfate sodium (DSS), a nongenotoxic sulfated polysaccharide (16, 17). Acute and chronic colonic inflammation can be induced eventually leading to dysplasia followed by colorectal cancer. Penetration correlates to duration of inflammation produced either by recurrent administration alone (17, 18) or by brief exposure in mice with genetic traits that amplify and perpetuate colitis (6, 7, 11). DSS-induced colitis has been attributed to direct metabolic epithelial toxicity, augmented by mucosal intrusion of enteric microbiota and their products, with resultant inflammation predominantly driven by innate rather than adaptive immune cell types (6, 12, 16, 19).

Several studies have addressed potential carcinogenesis-promoting mechanisms in colitis, including repair and regeneration of the epithelial barrier, the innate immune response, role of cytokines, and oxidative DNA damage at the local sites of inflammation in the colon (19–22). However, we are unaware of studies that have determined whether intestinal inflammation affects the level of systemic genotoxicity, such as in the peripheral blood. Accordingly, this study was developed to characterize and quantify the association of systemic DNA damage in acute and chronic colitis by both DSS administration and in models of spontaneous chronic immune colitis.

Materials and Methods

Animals. C57BL/6Jp<sup>+/p</sup> (3–4 months), Go<sub>a</sub>2<sup>−/−</sup> (B6/129Sv background; 3 months; ref. 9), and interleukin (IL)-10<sup>−/−</sup> (C3H/HeJ background; 3 or 6 months) were housed in the University of California at Los Angeles Department of Laboratory and Animal Medicine under specific pathogen-free conditions, autoclaved bedding and food, with standard rodent chow diet, acidified drinking water, and 12:12 light/dark cycle. All mice were bred at University of California at Los Angeles, except IL-10<sup>−/−</sup> and C3H/HeJ, which were purchased from The Jackson Laboratory.

Induction of chemical colitis. Experimental colitis was induced with 3% (w/v) DSS (Fisher Scientific; MW 40,000) dissolved in acidified drinking...
water (changed daily) ad libitum for three cycles. One cycle consisted of 7 days of treated water followed by 14 days of normal drinking water. Acute colitis was defined as a 7-day treatment, and chronic colitis was defined as any further treatment including remission periods. Control animals received sterile acidified water only. Symptoms (weight loss, stool consistency, and gross bleeding) were recorded daily for calculation of disease activity index (23).

**Blood collection.** Peripheral blood was collected from experimental mice via the facial/mandibular vein with a 5 mm lancet (Braintree Scientific) into EDTA-coated collection tubes (Braintree Scientific). For the comet assay, blood was immediately diluted 1:1 in PBS/10% DMSO and frozen at -80°C until further analysis. Freshly collected blood was immediately processed for all other assays. Identical blood samples were used for genotoxic endpoints as well as for cytokine expression.

**Alkaline comet assay.** To detect single- and double-stranded breaks as well as alkali-labile sites in DNA, the alkaline comet assay was done as described previously (24). Frozen blood was further diluted 1:15 in PBS before further preparation. After lysis and electrophoresis, gels were stained with SYBR Gold (Molecular Probes) and visualized under a fluorescent microscope (Olympus AX70) at ×10 magnification. Comet images were analyzed with the CAPS image analysis program. The olive tail moment, which represents both tail length and fraction of DNA in the tail, was used for data collection and analysis, in which apoptotic cells were excluded under previously proposed criteria (24).

**Determination of oxidative DNA damage.** The enzyme hOgg1-modified comet assay was used for determination of oxidative DNA damage (25). Following lysis, samples were washed in an enzyme wash buffer [40 mmol/L HEPES, 0.1 mol/L KCl, 0.5 mmol/L EDTA, 0.2 mg/mL bovine serum albumin (pH 8.0)] and then incubated at 37°C for 10 min in either control (buffer with no hOgg1) or enzyme-treated (buffer with hOgg1) solutions according to the manufacturer's recommendations (New England Biolabs). Both control and enzyme treated gels were then placed in electrophoresis buffer and processed identically to the alkaline comet assay.

**Immunofluorescence.** Peripheral blood was incubated in Buffer EL (Qiagen) on ice to remove erythrocytes. Samples were then processed on coverslips essentially as described elsewhere (26). Briefly, after fixation, permeabilization, and blocking, cells were incubated with mouse anti-phospho-γ-H2AX S139(P) at 1:500, mouse anti-8-oxoguanine clone 4135 at 1:250, or rabbit anti-nitrotyrosine at 1:200 (all from Upstate) followed by goat anti-mouse (1:250, or rabbit antihuman secondary antibodies identical to the procedures described above. Coverslips were mounted with Vectashield with 4′,6-diamidino-2-phenylindole (Vector Laboratories). Images were captured with CytoVision (Applied Imaging) connected to a Zeiss Axioplan 2 microscope. At least 125 cells were counted and cells with four more than distinct foci in the nucleus were considered positive for γ-H2AX (26). Apoptotic cells, which are distinguishable due to presence of 10-fold the number of nuclear foci in damaged cells (27), were not included in analyses.

**Paraffin sections (5 μm) of colons from IL-10−/− and wild-type controls were microwaved in 10 mmol/L citrate buffer (pH 6) for 10 min for antigen retrieval, blocked, and then incubated with anti-8-oxoguanine or anti-nitrotyrosine followed by secondary antibodies identical to the procedures described above.**

**In vivo micronucleus assay.** Micronuclei formation was determined in peripheral blood erythrocytes to assess chromosomal instability. Similar to a previously proposed method (28), 3 μL whole blood was spread on a microscope slide and stained in modified Wright-Giemsa solution (Sigma-Aldrich). Micronuclei were counted and scored with an Olympus AX70 at ×100 following previously proposed criteria (29). At least 4,000 mature erythrocytes were counted per mouse, and the frequency of micronuclei formation was calculated as number of micronucleated erythrocytes per 1000 normochromatic erythrocytes.

**Results**

**Evaluation of experimental colitis.** The disease activity index is the average combined score of weight loss (0–4), stool consistency (0–4), and bleeding (0–4) used to score clinical symptoms (23). DSS-treated mice showed rectal bleeding starting day 4 in cycle 1, represented by the increase in the disease activity index compared with nontreated animals (Fig. 1). However, the onset of severe symptoms came earlier in the second and third cycles of treatment due to chronic inflammation, even after 14-day remission periods. Bleeding and diarrhea ceased as soon as treatment was stopped during remission and no mortalities were observed after three cycles of treatment. Food intake was also not affected throughout the study and significant weight loss was only apparent during the end of the second and third cycle.

**DSS treatment causes DNA single- and double-stranded breaks in peripheral leukocytes.** Single- and double-stranded breaks as well as alkali-labile sites in DNA of peripheral leukocytes were measured in terms of the mean olive tail moment with the alkaline comet assay (Fig. 2). Whereas mean olive tail moments of nontreated mice remained low throughout each cycle of treatment, DSS-treated mice showed significantly higher olive tail moments at the end of each cycle (P < 0.01). After each remission period of 14 days, levels of DNA damage decreased most likely due to DNA repair. The hOgg1-modified alkaline comet assay was also used to detect oxidative base damage. Ogg1 primarily recognizes and removes 8-oxoguanine through a base excision repair pathway as well as 8-oxoadenine, fapy-guanine, and methyl-fapy-guanine (32). Mean olive tail moments were higher when incubated with hOgg1 after treatment cycles in treated mice compared with hOgg1-incubated nontreated mice (P < 0.05), indicating the presence of oxidized base damage. When compared with levels before cycle 1, hOgg1-incubated DNA from DSS-treated mice was significantly higher at every following time point (P < 0.01; data not shown). Levels of DNA damage increased with each treatment cycle especially when including oxidative base damage, indicating damaging effects of acute and, more significantly, chronic inflammation. A small number of apoptotic cells with extensive...
DNA fragmentation were apparent after treatment cycles but were not included in calculation of mean olive tail moments.

Presence of DNA double-stranded breaks alone was confirmed with immunofluorescence of γ-H2AX (Fig. 3A). In response to double-stranded breaks, H2AX is phosphorylated (γ-H2AX) in a 2-Mbp region flanking the double-stranded break within 15 min (27). Percentage of cells positive for nuclear foci increased dramatically in the DSS-treated group after the first 7-day acute treatment (P < 0.01) compared with nontreated animals. Although not as dramatic, percent positive cells remained elevated over nontreated animals until end of treatment (P < 0.05). Efficient DNA double-stranded break repair may be activated, decreasing the presence of foci in chronic inflammation due to the severely damaging nature of double-stranded breaks compared with oxidative base damage or single-stranded breaks.

**DSS-induced inflammation is clastogenic to erythroblasts.** The *in vivo* micronuclei assay was carried out in mature normochromatic erythrocytes circulating in the peripheral blood to determine chromosomal damage to erythroblasts (Fig. 3B). The incidence of micronuclei is commonly used as an index of cytogenetic damage, including chromosome breaks, spindle abnormalities, or structurally abnormal chromosomes, most frequently in erythroblasts/erythrocytes from peripheral blood or bone marrow (29). Mature micronucleated normochromatic erythrocytes represent the final developmental stage of erythroblasts containing micronuclei stemming in the bone marrow and thus permit the study of both generation and elimination of micronucleated erythrocytes (33).

Micronuclei formation was significantly induced after the first cycle of treatment in DSS-treated animals (P < 0.01) compared with nontreated animals and was further induced after the second and third cycles compared with both nontreated animals and levels before cycle 1 (P < 0.01). Similar to patterns seen in the results of the alkaline comet assay, micronuclei formation decreased after remission periods and increased after each cycle of treatment. This indicates clearance of micronucleated erythrocytes by the spleen followed by induction during treatment periods. Starting before cycle 3 time point, micronucleated erythrocyte levels were slightly elevated even in nontreated animals most likely due to the effects of repeated blood draws and consequentially high rate of erythropoiesis.

**DSS treatment modulates mRNA expression of cytokines in peripheral blood.** Systemic inflammation due to DSS treatment was shown by cytokine gene expression in the peripheral blood of treated animals. Leukocytes circulating in the periphery mounted a strong Th1 response characterized by up-regulation of TNF-α, MCP-1, and IFN-γ particularly after the first cycle of treatment (Fig. 4). TNF-α transcript levels followed DNA damage patterns of
increasing after each 7-day treatment cycle and then decreasing after each 14-day remission period. MCP-1 and IFN-γ transcript levels increased after the first cycle and then decreased after the remission period, where they remained low until increasing once again in the third cycle, showing a delayed secondary induction compared with TNF-α. Transforming growth factor-β, an anti-inflammatory cytokine, was also modulated similarly to MCP-1 and IFN-γ. DSS treatment induces both a Th1 response and an anti-inflammatory response over the acute and chronic phases of treatment in the peripheral blood.

**DNA damage is observed in genetic models of mucosal inflammation.** To further determine whether systemic genotoxicity is a general consequence of colitis, we measured DNA damage in two genetic models of mucosal inflammation without the use of DSS. We examined Goi2−/− mice at age 3 months (chronic active inflammation with neoplastic changes in colon) and IL-10−/− at age 3 and 6 months (in which mice have subclinical disease with minimal histologic inflammation and active disease and inflammation with mild epithelial hyperplasia, respectively; Fig. 5A). Single and double DNA strand breaks were significantly higher (P < 0.01) both in Goi2−/− mice compared with age-matched Goi2+/+ mice without clinical symptoms and in IL-10−/− mice with subclinical inflammation compared with age-matched IL-10+/+ mice using the comet assay (Fig. 5B). We then hypothesized that IL-10−/− mice with severe mucosal inflammation would have greater DNA damage than those with subclinical inflammation. These mice indeed showed higher levels of strand breaks than IL-10−/− mice with subclinical inflammation (P < 0.01) compared with those seen in Goi2−/− mice. Oxidative base damage, however, seemed only apparent in Goi2 mice as measured by hOgg1 incubation. DNA double-stranded breaks measured by γ-H2AX immunofluorescence (Fig. 5C) were also elevated in both Goi2−/− and IL-10−/− and Goi2−/− and IL-10+/+ mice, respectively, although only statistically significant in Goi2−/− mice (P < 0.05), and IL-10−/− mice with severe mucosal inflammation (P < 0.05). Finally, micronucleus induction in erythroblasts were also significantly elevated in Goi2−/− mice compared with Goi2−/+ mice (P < 0.01) and elevated but not statistically significantly elevated in IL-10−/− versus IL-10+/+ mice (Fig. 5D). Systemic genotoxicity can therefore be incurred by several modes of inflammation independent of DSS administration.

**Intestinal inflammation induces reactive oxygen species-mediated oxidative stress and DNA damage.** To determine potentially causative species of oxidative stress due to intestinal inflammation, peripheral leukocytes from DSS-treated mice (7 days, 3% w/v) and IL-10−/− mice (6 months) were isolated and stained for 8-oxoguanine or nitrotyrosine (Fig. 6A and B). 8-oxoguanine is an oxidative DNA lesion formed by reaction of hydroxyl radicals, metal hydroperoxides, or peroxynitrite with DNA, causing G:C to T:A transversions during replication (34). Nitrotyrosine is a biochemical marker for NO-induced peroxynitrite formation involving reactions with reactive oxygen and nitrogen species resulting in nitrative damage to proteins (35). DSS-induced inflammation caused a significant increase in both 8-oxoguanine and nitrotyrosine (P < 0.01) in peripheral leukocytes, as did those isolated from IL-10−/− mice (P < 0.05). Colon sections from IL-10−/− mice also showed 8-oxoguanine residues mostly in the nuclei of the surface epithelial cells as well as in infiltrating inflammatory cells within or near the lamina propria as “focus” like or pan-nuclear staining (Fig. 6C). Nitrotyrosine residues were also present.

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**Figure 4.** Quantitative real-time PCR of cytokines in peripheral blood. Expression levels of cytokines were determined only in DSS-treated mice (n = 10). Mean ± SE of gene expression divided by TATA-binding protein (Tbp) expression. A. Transcript levels of TNF-α divided by TATA-binding protein. B. Transcript levels of MCP-1 divided by TATA-binding protein. C. Transcript levels of IFN-γ divided by TATA-binding protein. D. Transcript levels of transforming growth factor-β divided by TATA-binding protein. Statistical significance was determined by nonparametric one-way ANOVAs with Dunn’s multiple comparison test. *, P < 0.05; **, P < 0.01.
in the cytoplasm of epithelial cells and inflammatory cells, whereas no immunoreactivity was observed in wild-type mice.

**Discussion**

Previous studies have established the role of inflammation-derived oxidative DNA damage to inflammatory and surrounding epithelial cells only at the localized sites of inflammation in the colon. Our study shows for the first time that this damage extends beyond the site of inflammation to circulating leukocytes and erythroblasts in the bone marrow, manifesting a systemic effect, and correlating with oxidative damage found in inflammatory tissue.

Genotoxicity to peripheral leukocytes was evident in terms of both single- and double-stranded breaks to DNA accompanied by oxidative base damage while chromosomal aberrations took place in erythroblasts. Such findings were observed in both acute and chronic phases of chemical colitis induced by DSS administration and untreated Gai2−/− and IL-10−/− mice undergoing spontaneous immune colitis. Moreover, in IL-10−/− mice, which are notable for a delayed onset of colitis, genotoxicity was further elevated in mice that had proceeded to a stage of clinically active colitis versus those with subclinical inflammation. Markers of reactive oxygen species-derived oxidative stress showed presence of 8-oxoguanine and nitrotyrosine in peripheral leukocytes of DSS-treated mice and...
IL-10−/− mice, representing possible mechanisms of genotoxicity and correlating to oxidative damage seen in the colon. Accordingly, the present study reveals that systemic genotoxicity is a prevalent feature of subclinical, acute, and chronic colitis.

In DSS-treated mice, repair of DNA damage was observed during remission periods represented by a decrease in damage markers. However, the extent of repair appeared slightly less in the last remission due to increasing severity of chronic inflammation. Despite increasing severity of inflammation, double-stranded breaks remained only slightly elevated over nontreated animals, which may imply efficient repair in comparison with single-stranded breaks and oxidative damage. DSS administration also induced systemic distribution of cytokines as evidenced by modulation of transcript levels in peripheral blood. Interestingly, TNF-α was up-regulated during treatment and down-regulated during remission, mirroring patterns seen in genotoxicity to leukocytes. Similar to previous cytokine studies in the colons of DSS-treated mice (20), features of both Th1 and Th2 activity were observed systemically in the peripheral blood, leading to chronic activation of immune cells. The decrease in MCP-1 and IFN-γ expression after the first cycle of treatment may be explained by a shift toward higher expression of Th2 cytokines and a decrease in selective Th1 cytokines as recently documented (36) in DSS-treated mice. Chronic DSS treatment mimics IBD with similar cytokine profiles showing dysregulated and imbalanced immunologic responses to commensal bacterial antigens. Dysregulated and polarized cytokine production play key roles in enhancing chronic inflammation and tumorigenesis through signaling release of promotor mediators (37).

The present study shows that both chemical and genetic/immune models of inflammation-mediated carcinogenesis not only parallel the inflammation to dysplasia to cancer sequence of human IBD but also manifest inflammation-associated oxidative stress in the colon as seen in ulcerative colitis and Crohn’s disease. Unlike other colitis-associated neoplasia models using genotoxic colon carcinogens as initiators of neoplasia (azoxymethane or 1,2-dimethylhydrazine), DSS itself is not a mutagen nor genotoxic (38). However, it has been shown to both directly and indirectly activate macrophages and other inflammatory cells (16, 39), a central feature of genetic models of immune colitis (8–11). Thus, carcinogenesis arising in these settings is solely a manifestation of chronic inflammation. The prominent mucosal and systemic activation of macrophages, neutrophils, eosinophils, and other effectors in DSS-induced colitis, genetic immune colitis (and in active disease of patients with IBD) is a potential source of oxidative stress. This
may cause oxidative and nitrative damage locally through oxidative burst and through release of cytokines that induce receptor-mediated reactive oxygen species production by target cells. Microsilastability was identified in tumors in colons of DSS-treated wild-type mice and more so in Msh2−/− mice (40). DSS treatment also induced 8-oxoguanine residues in mouse colonic mucosa (22), suggesting oxidative damage directly at the site of inflammation. Notably, this observed systemic genotoxicity is a secondary effect of DSS treatment, that is, the consequence of systemic inflammation and inflammation-associated oxidative stress. In agreement with these findings, we have shown 8-oxoguanine and nitrotyrosine formation in the surface epithelium and inflammatory infiltrate of IL-10−/− colons as well as in peripheral blood of IL-10−/− and DSS-treated wild-type mice, indicating systemic presence of peroxynitrite and reactive oxygen and nitrogen species.

We envision two nonexclusive processes linking local inflammation and systemic genotoxicity. First, locally activated innate immune cells may release reactive species inducing formation of other reactive species such as hydroxyl radicals and NO-derived peroxynitrite, damaging emigrating resident leukocytes that then circulate into the periphery. Alternatively, inflammatory cytokines achieve biologically significant systemic levels, on which they induce autonomous, cytokine-receptor mediated production of free radicals (and genotoxic damage) in remote leukocyte populations. Both scenarios are possible, as we observed proinflammatory cytokines throughout DSS treatment in the peripheral blood and oxidative DNA damage and nitrotyrosine formation in circulating leukocytes. Similarly, micronuclear formation in the erythroblasts of the bone marrow in our study may have been a result of activated T cells that are part of the normal recirculating lymphocyte pool circulating into the bone marrow and leading to oxidative damage. Accumulation of single- and double-stranded breaks can sequentially lead to chromosome breaks and micronuclei formation (41).

In addition, biological processes affected by inflammation may also determine the fate of cells bearing genotoxic damage. Because inflammatory mediators elicit both epithelial cell proliferation and antiapoptotic signals, epithelial cells in chronic inflammation are at particular risk to DNA damage leading to fixation of mutations that may not be properly repaired and removed (22). In DSS colitis, oxidative DNA damage was positively correlated with apoptosis in the small intestine but not the large intestine (42). This biological difference may contribute to the relative susceptibility to cancer progression in the large intestine. Although the mechanism of this differential induction of apoptosis is uncertain, genotoxic stress induces expression of ligands for the NKG2D receptor (43). This receptor is differentially expressed on resident CD8+ T cells and natural killer cells of the small versus large intestine and is a potent inducer of antiepithelial cytotoxicity in this intestinal region (44). Finally, the possibility of reciprocal regulation of inflammation and DNA repair pathway elements is an emerging area of investigation (45).

In summary, intestinal inflammation is associated with systemic genotoxicity through single and double DNA strand breaks, oxidative DNA damage, protein nitration, and micronucleus formation. We propose that elements of the inflammatory response including reactive oxygen species-derived oxidative stress are responsible for the observed systemic genotoxicity, although the exact cell types and inflammatory products responsible remain to be defined. Previous studies have observed oxidative base damage, microsilastability, and gene mutations directly in the colonic mucosa of both human IBD and experimental murine colitis. Here, we highlight that systemic DNA damage accompanied by systemic inflammation is an early event involved in the promotion of genic instability. Such systemic genotoxicity may be a biologically relevant and sensitive biomarker of one process contributing to inflammation-associated carcinogenesis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
Received 11/24/08; revised 3/23/09; accepted 4/6/09; posted online 6/1/09.

Grant support: NIH grants ES09519 (R.H. Schiestl), DK46763 (J. Braun), and CA016042 (Jonsson Comprehensive Cancer Center). Jonsson Comprehensive Cancer Foundation (R.H. Schiestl and J. Braun), and Crohn’s and Colitis Foundation of America (J. Braun).

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We thank Nagesh Rao for technical assistance with the micronuclear assay.

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