Increased p53 Mutation Load in Noncancerous Colon Tissue from Ulcerative Colitis: A Cancer-prone Chronic Inflammatory Disease

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Abstract

Ulcerative colitis (UC) is a chronic inflammatory disease that produces reactive oxygen and nitrogen species and increases the risk of colorectal cancer (CRC). The p53 tumor suppressor gene is frequently mutated in UC-associated dysplastic lesions and CRC. We are exploring the hypothesis that p53 mutations in the nontumorous colon tissue in noncancerous UC cases indicate genetic damage from exposure to exogenous and endogenous carcinogens and may identify individuals at increased cancer risk. We are reporting, for the first time, the frequency of specific p53 mutant alleles in nontumorous colon tissue from donors either with or without UC by using a highly sensitive genotypic mutation assay. Higher p53 mutation frequencies of both G:C to A:T transitions at the CpG site of codon 248 and C:G to T:A transitions at codon 247 were observed in colon from UC cases when compared with normal adult controls (P = 0.001 and P = 0.001, respectively). In the UC cases, higher p53 codon 247 and 248 mutation frequencies were observed in the inflamed lesional regions when compared with the nonlesional regions of their colon (P < 0.001 and P = 0.001). The colonic nitric oxide synthase-2 activity was higher in UC cases than in non-UC adult controls (P = 0.02). Our data are consistent with the hypothesis that a higher frequency of p53 mutant cells can be generated under oxidative stress in people with UC. The increased frequency of specific p53 mutated alleles in noncancerous UC colon tissue may confer susceptibility to the development of CRC in an inflammatory microenvironment.

Introduction

An increased risk of CRC6 has been reported among the patients with UC, a chronic inflammatory disease (1, 2). The colons of UC patients show uniform and continuous inflammation of the mucosa. The inflammatory reaction attributable to neutrophil infiltration causes epithelial damage, including multiple lesional regions of ulceration and hemorrhage. Whereas adenomatous polyps are considered to be the major precursor of sporadic CRC (3), UC-associated neoplasm involves the development of epithelial dysplasia that may affect large regions of mucosa (4). Chromosomal instability, e.g., loss of arms and centromere gains, have been reported in UC colon that may generate a mutator phenotype and aid in cancer development (5). A genetic and epigenetic model, involving both the activation of oncogenes such as ras and the inactivation of tumor suppressor genes such as APC and p53, in the development of the majority of CRC has been proposed (6–8). In addition to the differences in the histology and morphology of the precursor neoplasm, the frequency and timing of occurrence of these genetic alterations were reported to differ remarkably in sporadic and UC-associated CRC (9). A several-fold lower prevalence of the mutation in the ras oncogene and the APC tumor suppressor gene was reported in UC-associated CRCs when compared with sporadic CRC (7, 10, 11). The occurrence of both ras and APC mutations also is unusual in neoplastic and preneoplastic colon in UC (12, 13). Alterations in the p53 tumor suppressor gene, a late event in the molecular pathogenesis of sporadic CRC (14), were reported in the earliest characterized UC-associated dysplastic lesions (15, 16) and may likely precede dysplasia (17). Mutations in p53 have been reported in the colonic lavage fluid of UC patients (18). Recently, Kinzler and Vogelstein (19) proposed the concept of the “landscaper defect.” According to this concept, the abnormal microenvironment, produced by defective stromal cells, influences the neoplastic transformation of epithelial cells in UC and juvenile polyposis syndromes.

Although NO is an important bioregulatory agent and signaling molecule that mediates a variety of physiological functions such as vasodilation, neurotransmission, host defense, and iron metabolism, increased NO production may contribute to the pathogenesis of a number of chronic diseases including cancer (20–24). Accumulation of nitrotyrosine in the inflamed mucosa of patients with UC indicates that NO production and the formation of peroxynitrite are involved in the pathogenesis of this disease (25). Our previous investigation of primary human colon tumors established a statistically significant positive correlation between the NOS2 activity in tumors and the frequency of G:C to A:T transitions at the CpG sites in the p53 tumor suppressor gene (23). NO also may functionally interact with p53 in modulating tumor progression. For example, when compared with isogenic control cells, NOS2-expressing human cancer cells with wild-type p53 have a reduced tumor growth in athymic nude mice. In contrast, NOS2-expressing cancer cells with mutated p53 have an accelerated tumor growth that was associated with increased vascular endothelial growth factor expression and neovascularization (24). Whereas a number of studies implicate NO in the pathogenesis of cancer (reviewed in Refs. 26 and 27), other reports indicate the antitumor properties of NO (22, 28). The timing, concentration, flux, microenvironment, and genetic alterations of the cells exposed to NO were suggested as the basis of a varying and sometimes opposing role of NO in carcinogenesis. In this study, we present data consistent with the hypothesis that the p53 mutation load is increased in the lesional inflammatory epithelium of the cancer-prone condition, UC.

Materials and Methods

Nine paired colon endoscopic biopsies from nine patients with UC representing active lesions and nonlesional tissues were available for analysis. In addition, nine other active lesional samples without their nonlesional matching pair from nine UC patients were also used. Ten large colon postmortem...
representative tissues from 10 normal donors (control samples) also were included. The tissues were obtained from the Cooperative Human Tissue Network (Philadelphia, PA) or the University of Maryland, Department of Pathology (Baltimore, MD).

**NOS2 Immunohistochemistry.** The immunohistochemical staining procedure was performed as reported (29), using primary anti-NOS2 monoclonal antibody (Transduction Laboratory, Lexington, KY) at a dilution of 1:125. Positive controls were represented by lung cancers known to express NOS2. Negative controls were produced by substituting the primary antibody with nonimmune rabbit serum. Interpretation was done with no prior knowledge of NOS2 enzymatic activity, as measured biochemically and described below.

**Analysis of p53 Codons 247 and 248 for Missense Mutations by MspI-RFLP/PCR.** As described previously in detail (30, 31), the genomic DNA that was digested exhaustively with the MspI restriction enzyme contained approximately 3–3.5 × 10^7 initial p53 copies. Thirty copies of a MS were added as an internal control. The samples were enriched in sequences with a mutated MspI recognition sequence 14067–14070 that spans the third position of codon 247 and the entire codon 248 by agrose gel electrophoresis and gel isolation of a 380–500-bp fragment population. These DNA preparations contain 462-bp MS and the predicted, mutated 468-bp fragments that extend from the flanking 5′ MspI site (nucleotide residue 13768) to the flanking 3′ MspI site (nucleotide residue 14235).

A final 101-bp exon VII fragment that extends from residues 13999 to 14099 and contains codons 247 and 248 was amplified from the above enriched DNA preparation in two rounds of amplification using *Pyrococcus furiosus*-DNA polymerase (Stratagene, La Jolla, CA) and Taq-DNA polymerase (Perkin-Elmer, Foster City, CA). The RFLP/PCR products were cloned into Agt10, and the phages were plated on Escherichia coli C600 Hill. For each of the samples, a total of 1200–1600 plaques were grown on 10–12 different Luria broth agar plates. Each plate contained 125–150 plaques. These plaques were lifted on plaque screens and hybridized separately with a total of 14 copies. Thirty copies of a MS were added as an internal control. The samples were enriched in sequences with a mutated MspI recognition sequence 14067–14070 that spans the third position of codon 247 and the entire codon 248 by agrose gel electrophoresis and gel isolation of a 380–500-bp fragment population. These DNA preparations contain 462-bp MS and the predicted, mutated 468-bp p53 fragments that extend from the flanking 5′ MspI site (nucleotide residue 13768) to the flanking 3′ MspI site (nucleotide residue 14235).

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Fig. 1. A composite of H&E in lesional and nonlesional UC tissues (A and C) with anti-NOS2 immunohistochemistry results (B and D). Neutrophils in the lamina propria are seen in active lesions of UC with an involvement of the adjacent colonic glands (A). In sections D, reactive and regenerative glandular change was observed. The control samples were unremarkable except for one, which showed minor evidence of postmortem autolysis.

**Light Microscopy and NOS2 Immunohistochemistry.** The colonic lesional biopsies showed varying amounts of inflammation including neutrophilic infiltration in the lamina propria and the adjacent colonic glands with microabscess formation (Fig. 1A). In sections obtained from nonlesional biopsies, no active inflammation was present; only reactive and regenerative glandular change was observed (Fig. 1C). The control samples were unremarkable except for one, which showed minor evidence of postmortem autolysis.

**Results.**

**Measurement of NOS2 Activity.** Tissue fragments (<500 mg) were crushed with a pestle and mortar under liquid nitrogen and homogenized with a PowerGen 125 or a Brinkmann PolytronR homogenizer in 1.5–2.5 ml of buffer A (50 mM HEPES, 1 mM DTT, 1 mM L-citrulline, 1 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 10 mg/l leupeptin, and 3 mg/l aprotinin, pH 7.4) at 0–4°C. Endogenous arginine was removed by adding Dowex AG 50W-X8 resin (~200 mg). The samples were centrifuged (15,000 × g; 4°C for 10 min), and the supernatants were used for determination of NOS activity by measuring the conversion of L-arginine to L-citrulline, as described previously (32). The results were expressed as pmol/min/mg protein.

The immunoreactivity of >2 (>25 to <50% reactivity) was observed in seven lesional and two nonlesional samples (Fig. 1, B and D). However, the pattern of expression was different. NOS2 immune expression was seen in the inflammatory cells of the lamina propria in lesional and the cytoplasm of colonic glands in nonlesional tissue in a similar pattern as described previously (33). The control samples showed similar colonic gland patterns of NOS2 expression in four samples, with two samples showing localization to inflammatory cells in the lamina propria.

**Analysis of p53 Mutation Load in Colon from UC Patients.** The p53 mutation load at codons 247 and 248 in nine lesional and nine paired lesional and nonlesional, nonneoplastic colon samples from 18 UC patients and 10 normal colon samples from non-UC postmortem control were analyzed. For each of the samples, 1200–1600 plaques were analyzed on 10–12 Petri dishes. Fig. 2 shows representative membranes with the identified mutant plaques in one of several Petri dishes for each sample set. More than 50% of the UC cases (n = 18) showed a higher mutation frequency of G-to-A transitions at the CpG...
site of codon 248 when compared with non-UC controls ($P < 0.001$; $n = 10$; Mann Whitney nonparametric analysis; Fig. 3A). The C-to-T transitions at the third base of codon 247 also were observed with higher mutation frequencies in the UC cases than the non-UC controls ($P = 0.001$; Fig. 3B).

An increased $p53$ mutation load in more than half of the UC cases indicates a possible role of inflammation-induced oxidative stress in the induction of these mutations. Therefore, we compared the $p53$ mutation load in codons 247 and 248 in matched pairs of inflamed lesional and nonlesional colon in a subset of nine UC patients. Interestingly, both G-to-A and C-to-T transitions were found with higher mutation frequencies ($P < 0.001$ and $P = 0.001$) in inflamed lesonal regions of the colon when compared with nonlesional regions (Fig. 4).

**NOS2 Activity in UC Cases and Non-UC Controls.** A number of studies have shown that NO can induce mutations by either direct or indirect pathways (34, 35). To determine whether there is a correlation between NOS2 activity and the frequency of $p53$ mutation in UC cases and non-UC controls, we measured the NOS2 activity in these samples. The mean of NOS2 activity in the colon tissue was higher in UC cases than in the non-UC controls ($P = 0.02$; Fig. 5). Furthermore, in 7 of the 18 UC samples, high levels of NOS2 activity strongly correlated with NOS2 immunopositivity.

**Discussion**

Recent studies have linked chronic inflammation with somatic mutations and an increased risk of cancer (reviewed in Refs. 26, 27, and 36). The positive association of hepatitis (after hepatitis B or C viral infection) and hepatocellular carcinoma (37, 38); UC and colorectal cancer (1, 2); pancreatitis and pancreatic cancer (39); schistosomiasis and bladder cancer (40); and *Helicobacter pylori* infection and gastric cancer (41) indicates that chronic inflammation is a cancer-prone state. However, the molecular mechanisms by which inflammation contributes to carcinogenesis and tumor progression are not defined clearly.

Mutation in the $p53$ tumor suppressor gene is the most common...
The increased frequency of a mutated p53 allele in nontumorous tissue in cancer-prone inflammatory disease may be considered as a marker for increased susceptibility to cancer. In the present study, the existence of a high frequency of specific p53 mutated alleles in inflamed lesional regions and not in the nonlesional regions of the colon in UC patients is consistent with the hypothesis that reactive species produced during inflammation can induce these mutations. However, the exact mechanism by which sustained oxidative stress, generated during chronic inflammation, induces these mutations is not understood fully.

A constant flux of reactive species that principally include O$_2^\cdot$, H$_2$O$_2$, OH, NO, NOx, and OONO$^-$ can be generated during chronic inflammation. An increased level of these reactive species and their direct and/or indirect effects can alter DNA. A variety of bp changes are observed after exposure to reactive oxygen species that depend largely on the mutation assay system and the source of active oxygen (36, 42).

The deamination of 5-methylcytosine has been argued to be a major mechanism for the induction of G:C to A:T mutations at CpG dinucleotides in DNA (reviewed in Refs. 43 and 44). NO produced during inflammation may cause both deamination and oxidative damage to DNA. Macrophages activated with E. coli, lipopolysaccharides, and IFN-γ produce NO and reactive oxygen species and cause deamination and oxidation of DNA bases (45). The treatment of TK6 human lymphoblastoid cells with NO deaminated guanine and adenine and induced mutations at the hypoxanthine phosphoribosyltransferase and thymidine kinase gene loci (46). NO also facilitates 5-methylcytosine deamination (47), and a positive correlation was observed between higher NOS2 activity and increased p53 G:C to A:T transitions at CpG sites in colon carcinoma (23), a finding consistent with the hypothesis of enhanced NO-induced deamination under hypoxic conditions. Autoxidation of NO under low O$_2$ tension leads to the generation of nitrosative species such as N$_2$O$_3$. The nitrosative properties of N$_2$O$_3$ are suggested to be responsible for the deamination of bases (46, 47). On the basis of the required ratio of NO:O$_2$, an increased formation of nitrosative species such as N$_2$O$_3$ is expected under hypoxic conditions, e.g., in the tumor microenvironment and in the presence of a high NO concentration. In contrast, NO autoxidation produces less nitrosative intermediates in normoxic conditions including inflammation. Our data are consistent with this latter possibility in that C-to-T mutations were not found at the CpG site of codon 248 in the inflamed colon of UC cases. A possible mechanism for the high frequency of C-to-T transitions at codon 247 (a non-CpG site with an unmethylated cytosine) could be the direct modification of cytosine by reactive oxygen species. Whereas both G-to-A (codon 248) and C-to-T (codon 247) transitions were observed with similar frequencies in the present study, only the codon 248 missense mutation produces an amino acid substitution. The presence of a silent mutation at codon 247 with almost the same frequency as the missense mutation argues against the clonal expansion of the mutated cells as an explanation of the increased number of the cells with p57 mutations.

NO and superoxide anions, produced by activated macrophages and neutrophils during chronic inflammation, may produce peroxynitrite (OONO$^-$). The detection of increased NOS2 overexpression in inflammatory cells of lamina propria in samples obtained from active UC lesions is not unusual. Under normal physiological conditions, OONO$^-$ may either rearrange and be excreted as nontoxic nitrate or form reactive species that possess a hydroxyl radical (OH$^-$)-like reactivity (48). Peroxynitrite also may generate 8-nitroguanine and 8-hydroxyguanine (49, 50). In addition, during normoxic conditions, NO may protect against oxidative DNA damage (reviewed in Ref. 51). Because NO reacts with superoxide radicals, the overall effect of this reaction might result in the quenching of superoxide anion radical toxicity. Furthermore, any reaction between OONO$^-$ and NO or O$_2$ limits the reactivity of OONO$^-$ by forming nitrogen dioxide (reviewed in Ref. 52).

The initiation of lipid peroxidation may be another pathway by which reactive oxygen and nitrogen species cause DNA base damage and mutation. Reactive oxygen and nitrogen species such as O$_2^\cdot$, H$_2$O$_2$, OH, and OONO$^-$, initiate and propagate lipid peroxidation (53). Some of the lipid-peroxidation intermediates can modify DNA by forming ethenobases or exocyclic adducts. Lipid peroxidation is described as an endogenous source for the formation of exocyclic adducts (54, 55), which induces a variety of bp changes. In the present sample set, ethenoguanine adduct levels in UC cases were not different from the levels in controls (data not shown).

Our results indicate that a substantial subset of patients with UC carry a high p53 mutation load. The high frequency of these mutations in the inflamed lesional tissue compared with the nonlesional regions of the same colon is consistent with the hypothesis of an inflammation-associated oxidative stress in the etiology of these p53 mutations. The complex chemistry of reactive oxygen and nitrogen species and their function (which depends on the ratio of different reactive species and several other factors, such as O$_2$ tension) makes it difficult to associate a single pathway in the generation of a high p53 mutation load in the inflamed colons of UC patients. However, the presence of a high frequency of mutated p53 alleles with G-to-A and C-to-T transitions, predominantly in the inflamed lesional portion of the colon of UC cases without cancer, suggests the involvement of multiple pathways involving reactive oxygen and nitrogen species that are produced during inflammation. On the basis of these and other data, prospective studies to determine whether UC cases with high p53 mutation loads are at increased risk of colorectal carcinoma are warranted.

Acknowledgments

We thank Dorothea Dudek for editorial and graphic assistance.

References


Fig. 5. NOS2 activity in colon of UC cases and non-UC controls.


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