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REVIEW

Oxidative stress and ulcerative colitis-associated carcinogenesis: studies in humans and animal models

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The chronic inflammatory bowel disease ulcerative colitis (UC) occurs commonly in the US and other Western countries, but its etiology is unknown. An association between UC and an elevated risk for colorectal cancer is well established. UC-associated colorectal carcinogenesis is probably driven by chronic inflammation, but the mechanism is unclear. The morphological development of UC-associated cancer differs from that of its sporadic counterpart. Similarly, detailed molecular analyses have indicated that whereas many of the genetic alterations observed in sporadic colon cancers also occur in UC-associated neoplasms, the timing and frequency of those changes in the setting of UC are different. These histological and molecular signatures may very well be reflective of an inflammation-driven carcinogenesis process in UC patients. Studies in animal models of UC have helped to shed light on the mechanisms of inflammation-driven colorectal carcinogenesis. The available evidence suggests that DNA damage caused by oxidative stress in the characteristic damage–regeneration cycle is a major contributor to colorectal cancer development in UC patients. Based on this concept, iron over-nutrition is proposed as a risk factor and dietary antioxidants as protective factors for UC and associated carcinogenesis.

Occurrence and etiology of ulcerative colitis

Ulcerative colitis (UC) is an idiopathic disease characterized by mucosal inflammation of the large bowel. Approximately 10 individuals per 100 000 per year are diagnosed with UC (1). The incidence of UC varies depending on geography, and is most common in Western countries, including the US (2). It is predominately a disease of late adolescence and early adulthood, with the peak of incidence occurring in the third decade of life (3). UC is more common in Caucasians, and the trend for gender has varied with the study and the population (1,2). The susceptibility of certain ethnic groups to UC suggests a strong genetic contribution to this disease, but it is also apparent that the environment plays an important role (4).

The etiopathogenesis of UC remains uncertain, but many factors have been proposed to be involved in the initiation and propagation of the chronic inflammatory response in UC patients. Those findings can be summarized by two major conceptions: (i) that UC is an aberrant response to commonly encountered environmental stimuli, or (ii) UC arises as a normal immune response to a persistent infection or altered colonic microflora (5,6). UC may very well arise from a combination of these scenarios.

Genetic factors: antigen presentation

An abnormal response to normally occurring factors would suggest a genetic predisposition to the development of UC. Genetic studies have indicated associations between UC and major histocompatibility complex (MHC) class II antigens within some populations (7,8). Three susceptibility loci for inflammatory bowel disease (IBD) on chromosomes 3, 7 and 12 have been identified by microsatellite marker-based linkage analysis (9). Candidate genes at these locations include *GNAI2*, which encodes an inhibitory G protein, the mucin gene *MUC3*, the genes encoding hepatocyte growth factor and the epidermal growth factor receptor, and the *MHC* locus.

Altered colonic barrier function

Intrinsic inabilities to regulate normal intestinal bacterial flora or to modulate the threat of intestinal infection may be UC susceptibility factors. An example would be a defect in intestinal barrier function, which would increase the exposure of intestinal cells to luminal contents. Indeed, the surface mucus layer is thinned in UC patients (10). A deficiency in mucin production, or altered mucin structure and lectin binding, could also compromise intestinal barrier function (11,12). Defects in short chain fatty acid metabolism may lead to decreased barrier function as well as epithelial cell starvation (13,14). Immunoglobulin dysfunction and proteolytic enzyme deficiency could also conceivably cause barrier breaches (5).

Immunological factors

The immune response of UC patients is characterized by a predominance of T_H2 cytokines, and the preferential activation of T_H cells by epithelial cells (5). Several transgenic and gene-targeting animal models of intestinal inflammation also suggest a failure or lack of proper immunoregulation in the etiopathogenesis of UC and IBD in general. Gene knockouts of *Il-10* and TGF-β, both of which encode anti-inflammatory cytokines, lead to the spontaneous development of chronic intestinal inflammation (15,16). Interestingly, knockouts of the pro-inflammatory cytokine *Il-2* also develop colitis, as do animals lacking *Tcr* (T-cell receptor) and *MHC II* (17,18).

Serum- and tissue-bound antibodies against a 40 kDa antigen believed to be tropomyosin react with colonic epithelial cells, as well as with several sites of systemic involvement in UC (19). Other groups have reported findings of anti-goblet cell serum antibodies (20), anti-endothelial cell antibodies (21), and perinuclear anti-neutrophil cytoplasmic antibody (pANCA) in UC patients (5). However, the role of autoimmunity in the pathogenesis of UC is unclear.

Abbreviations: CGH, comparative genomic hybridization; DALM, dysplasia-associated lesion or mass; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; iNOS, inducible nitric oxide synthase; MHC, major histocompatibility complex; MIN, microsatellite instability; NAC, *N*-acetylcysteine; NO, nitric oxide; NOS, nitric oxide synthase; RONS, reactive oxygen and nitrogen species; UC, ulcerative colitis.

Bacterial or viral infection

Several pieces of epidemological data suggest that infection may be involved in the initiation of UC. These include associations between UC and prenatal infection in the mother or postnatal infection in the child (22), seasonal variation in UC incidence (1), and the effectiveness of antibiotics in treating UC. The colons of UC patients have increased amounts of certain streptococci (23), and adhesive bacterial strains predominate in the fecal flora of UC patients (24). IBD patients produce larger amounts of anti-bowel bacterial antibodies in general (25). UC has also been associated with viral infection (26,27), but it is unclear if such observations are indications of causing factors.

Altered colonic microflora

Alterations in the number, activites or distributions of normal colonic luminal constituents may contribute to UC. The numbers and activity of sulfate reducing bacteria, as well as hydrogen sulfide levels, are elevated in the feces of UC patients. Hydrogen sulfide, produced from luminal sulfate by sulfate reducing bacteria, has been shown to impair short chain fatty acid metabolism (28,29). Animal models of colitis have also suggested a role of normal luminal bacteria in the induction of UC. Intestinal inflammation does not occur in many of the chemically induced and genetic animal models of colitis under germ-free conditions (6).

Histological and molecular pathogenesis of UC-associated colorectal cancer

Chronic UC is associated with an increased risk of developing colorectal cancer. The relative risk of colorectal cancer development in UC patients is 10-fold greater than in the general population (30–33). The risk of developing cancer, or its precursor lesion, dysplasia, increases exponentially with the duration of the disease (34). Indeed, surveillance for dysplasia and cancer is recommended for patients with UC for >10 years (35). Increasing extent of UC at diagnosis also correlates with greater risk of colorectal cancer. For example, individuals with pancolitis (UC involving the entire colon) are more likely to develop colorectal cancer than those with left-sided disease only (33,36).

The histopathogenesis of UC-associated colorectal carcinogenesis is widely believed to involve a step-wise progression from inflamed and hyperplastic epithelia, to flat dysplasia and finally adenocarcinoma (37). This is often contrasted with the adenoma sequence thought to give rise to sporadic colon cancer. The idea that cancer derives from a multistep carcinogenesis process, entailing sequential alterations at the molecular level that may underlie tissue-level changes, has gained support from studies on many different cancers (38–40). Similarly, UC-associated cancer is presumed to arise from an accumulation of genetic alterations in tumor suppressor genes, oncogenes and genes encoding DNA repair proteins, as well as an overall loss of genomic stability. Comparisons of the molecular alteration profiles of sporadic and UC-associated colorectal cancers have indicated subtle differences. The two types of colorectal cancer share alterations in many of the same genes and overall processes. However, the timing and frequency of the molecular genetic alterations in UC-associated cancers appear to be unique. These distinctive molecular profiles are presumed to result from different etiological factors and cellular environments.

Cytogenetic alterations, chromosomal instability and microsatellite instability (MIN)

Many studies have been performed in an effort to determine if abnormal DNA content, or aneuploidy, is a more reliable predictor of UC-associated cancer development than dysplasia. The results of prevalence studies on UC biopsy samples and archival specimens have shown that chromosomal abnormalities increase with the histological progression from normal to inflamed and regenerative epithelium, dysplasia and cancer. Regions of aneuploidy in the colons of UC patients are frequently associated with dysplasia, and possibly precede overt histological changes (41,42). Aneuploid tissue samples are more frequent in 'high-risk' patients with disease duration of >10 years, but aneuploidy has also been detected in colon samples of low-risk patients (42). Aneuploidy in non-cancerous mucosa adjacent to UC-associated cancers is greatly increased as compared with the mucosa of non-cancer patients, suggesting that carcinoma arises from a field of genetically abnormal epithelia (43).

Chromosomal instability is believed to contribute to aneuploidy and a variety of chromosome-level changes, including deletions, amplifications and translocations. Chromosomal instability is the most frequently occurring form of genomic instability in UC-associated cancers as revealed by studies using fluorescence *in situ* hybridization (FISH) and comparative genomic hybridization (CGH) (44,45). FISH analysis of biopsies from colectomy samples has shown that UC-associated carcinoma and dysplasia exhibit monosomies and polysomies. These abnormalities are frequently conserved between non-dysplastic and dysplastic epithelium, and between dysplasia and cancer. CGH analysis of biopsies from the same site revealed an increasing frequency of chromosome losses or gains from non-dysplastic epithelia to dysplasia and carcinoma. Conversely, CGH of samples from UC patients at low risk for carcinoma development (defined as disease of <8-years duration) revealed no chromosomal anomalies (44). The percentage of colectomy sites with chromosomal alterations increased with the histological progression to carcinoma, as did the number of alterations per site (45). These results suggest that chromosomal instability is an early event in the progression to UC-associated carcinoma, and may contribute to widespread aneuploidy and eventually dysplasia.

MIN is characteristic of a genome-wide deficiency in the faithful replication of repetitive DNA sequences. The frequency of MIN has ranged from 8 to 21% in UC-associated carcinomas and 13 to 19% in dysplasias (46–48). MIN has also been detected in inflamed and regenerative epithelia (48–50). Brentnall *et al*. detected MIN in 50% of non-dysplastic but chronically inflamed colonic mucosa samples, whereas the frequency was much lower in the study by Noffsinger *et al.* (8.9%: 25 out of 280 samples). MIN-positive, diploid cancers thus comprise a subset of UC-associated carcinomas, ~10–15%. The mechanistic role of microsatellite alterations in UC-associated carcinogenesis requires further study. The relatively high frequency of MIN in non-dysplastic, inflamed epithelia as compared with dysplasia suggests that MIN may be associated with chronic inflammation, perhaps oxidative stress. However, the majority of UC-associated lesions appear to emerge from a pathway involving chromosomal instability and aneuploidy. UC and the associated carcinogenic progression are characterized by accelerated telomere shortening (51). Conceivably, loss of telomere integrity could contribute to a

degeneration of chromosomal stability as well as changes in chromosome number.

Tumor suppressor gene alteration in UC-associated carcinoma and dysplasia

Tumor suppressor gene p53. Several studies have investigated the status of the p53 tumor suppressor gene and p53 protein expression during the progression to UC-associated carcinoma. *p53* is frequently altered in human cancer and is important in the cellular response to DNA damage due to exogenous and endogenous factors, including oxidative stress (52). p53 protein accumulation, which is associated with *p53* mutation as well as wild-type p53 over-expression, is frequently detected in UC dysplasias and carcinoma by immunohistochemistry (53–55). In addition, *p53* alterations have frequently been detected in non-dysplastic, regenerative epithelium and precede the development of UC-associated dysplasia and carcinoma (56). LOH of *p53* is common in UC-associated carcinoma and dysplasia. *p53* allelic loss was observed in ~70% of cancer cases and 45% of informative dysplastic lesions (54,55,57–59). *p53* LOH was also observed in as many as one-quarter of non-dysplastic, actively inflamed epithelia samples, as well as in indefinite-for-dysplasia samples (59,60).

Nearly 70% of UC-associated cancers and 20% of dysplastic lesions analyzed contained *p53* mutations (53,55,57). Brentnall *et al*. and Holzmann *et al*. detected *p53* mutations in non-dysplastic, normal epithelial samples from UC colectomy specimens at a frequency as high as 29% (61,62). The percentage of *p53* mutation-containing samples in these studies increased with the morphological progression to carcinoma, and, overall, *p53* mutation correlated with the degree of dysplasia (53,55,57,61,62). The majority of mutations involved exons 7 and 8. Transition mutations in *p53* codons 247 and 248 were prevalent in the inflamed mucosa of UC patients, more so in regions containing active lesions than those not containing such lesions (63). Codon 248 appears to be a hotspot for mutation in the p53 gene, but hotspots unique to UC-associated cancers have not been reported (53,54,62–64). Interestingly, the *p53* mutation spectrum in UC-associated lesions is dominated by transition mutations, which account for nearly 80% of the mutations (53,54,57,62,64). Base transitions can be caused by oxidative DNA damage: the intermediates of lipid peroxidation and alkylating agents can induce G to A mutations; C to T transitions may be attributable to the formation of 5-hydroxycytidine, or to the spontaneous or nitration-induced deamination of 5-methylcytosine at CpG sites (65,66). The early appearance of *p53* alteration makes it a clinically useful marker in the screening for UC-associated malignancy and in the assessment of cancer risk. *p53* alteration detection may also be applicable to non-invasive screening procedures. For example, *p53* mutation is detectable in colonic lavage fluid from long-standing UC patients (67).

Retinoblastoma gene (Rb) and p16^{INK4a}. The tumor suppressor gene Rb is often mutated or lost in epithelial tumors (68). Relatively few studies, however, have examined the role of *Rb* alteration in UC-associated carcinogenesis. In the largest study, *Rb* LOH was detected in about one-quarter of UC-patients with carcinoma, dysplasia-associated lesion or mass (DALM), or dysplasia (64). Another study found that *Rb* exhibits LOH in one-third of UC cancers, and in less than a quarter of dysplastic lesions (58). Loss of 13q, the site of the Rb locus, was observed in one out of four UC-associated cancers, but all lesions exhibited normal Rb immunostaining (57). Overall, *Rb* LOH was observed in 30% of UC-associated cancers and 20% of dysplasias studied (57,58,64).

The cyclin-dependent kinase inhibitor p16 is a component of the Rb tumor suppressor pathway (69). Earlier LOH studies of the p16 locus (9p21) showed a high rate of *p16* loss in dysplasias (66%), as well as in inflamed epithelia and adjacent normal epithelia (40 and 18%, respectively) (59,70). These findings contrast with those in sporadic colon cancer, in which *p16* is a less frequent target for inactivation (71). Methylation of the *p16* promoter has been observed in three-quarters of dysplastic or cancerous lesions, and in one-third of non-dysplastic samples analyzed (72). Alterations of *p53* and *p16* may be important early markers of carcinogenic progression in UC patients.

Adenomatous polyposis coli (APC) gene. The loss of *APC* is considered the initiating event of sporadic colorectal carcinogenesis (73). Mutant APC proteins have been detected in 17% of UC-associated dysplasia- or carcinoma-bearing patients (57,70,74). Other studies showed that 0–33% of cancers and 25–60% of dysplasias expressed mutant *APC* (57,74). Nearly 30% of dysplastic lesions and 59% of cancers exhibited *APC* LOH (58,59). Overall, 43% (15/35) of informative lesions showed allelic loss of *APC*. However, *APC* alteration was not detected in non-dysplastic, inflamed epithelia (58,59). In contrast to sporadic colorectal carcinogenesis, *APC* alteration is a relatively late event in the dysplasia sequence and occurs in a subset of UC-associated colorectal carcinomas.

Alterations of other tumor suppressor genes in UC-associated neoplasms. Losses at chromosome 18q are relatively rare events during UC-associated carcinogenesis. LOH of 18q, the site of the putative tumor suppressor gene Deleted in Colon Cancer (*DCC*) was observed in only 12% (1/8) of cancers and 33% of the dysplasia lesions, and was not detected in non-dysplastic, inflamed epithelia (59). Using Southern blots, Kern *et al*. found *DCC* LOH in 50% (2/4) of carcinoma cases (57). Alteration of another resident of 18q, Deleted in Pancreatic Cancer-4 (*DPC4*), was not detected in UC-associated carcinomas by direct sequencing or *in vitro* synthesized protein assay (75).

Tumor suppressor genes containing coding region microsatellite sequences may be subject to inactivation in MIN-positive cancers. The TGF-β1RII gene contains two coding region microsatellites, one of which (a poly A tract) is a frequent target of base deletion or insertion in MIN+ colon cancer cell lines. Seventeen percent (3/18) of MIN+ UC-associated lesions contained poly A tract mutations in *TGF-β1RII* in one study (in contrast, 81% of MIN+ sporadic colon carcinomas analyzed contained *TGF-β1RII* microsatellite mutations) (76). Conversely, 2% (1/43) of microsatellite-stable UC-associated lesions showed *TGF-β1RII* mutations. Microsatellite mutations of the IGFIIR gene have also been detected in UC-associated neoplasms (77), and in the gene encoding the transcription factor E2F-4. *E2F-4* contains a trinucleotide microsatellite repeat which contained mutations in 33% (4/12) of the UC-associated lesions analyzed (78).

K-ras oncogene

Like *APC* loss, activating mutations of the *K-ras* oncogene are common in the early stages of sporadic colorectal carcinogenesis. A total of seven studies on UC-associated colorectal carcinoma and dysplasia indicated a lower but significant frequency of *K-ras* mutation in UC-associated carcinoma. Overall, *K-ras* mutation was detected in 24% of UC-associated

lesions, including 24% of carcinomas and 23% of dysplasias (53,57,60,74,79–81). Chaubert *et al*. detected K-ras mutations in two out of seven (15%) samples of actively inflamed epithelia (53). *K-ras* mutation does seem to play a significant role in the later stages of UC-associated carcinogenesis, although studies on earlier histological changes are limited.

DNA repair genes: mismatch repair

Alterations in mismatch repair genes may contribute to the MIN+ subset of UC-associated carcinomas. Brentnall *et al*. (82) found germline splice site substitutions within exon 13 of *MSH2* in 26% (14/53) of patients with UC-associated carcinoma or dysplasia. This polymorphism was detected in 11% (4/36) of patients negative for UC, and in 9% (7/80) of normal patients. However, in a study by Noffsinger *et al.* (48), no relationship between the germline *MSH2* alteration and UC-associated dysplasia or carcinoma incidence, or the occurrence of the MIN phenotype, was observed. Pokorny *et al*. (83) found an association between certain *MLH1* gene haplotypes and UC. Genetic or epigenetic alterations of mismatch repair proteins, including *MLH1* promoter hypermethylation (84) and loss of MSH2 expression (47), may lead to high-level MIN in UC-associated lesions. On the other hand, low-level MIN is not associated with such mismatch repair gene alterations, although other mismatch repair genes such as *PMS1* and *MSH6* have not been studied.

Aberrant gene methylation

As mentioned above, hypermethylation and possible silencing of the $p16^{INK4a}$ gene occurred at a high rate in dysplasia and carcinoma in UC colectomy specimens (72). Hypermethylation of $p14^{ARF}$, encoding a modulator of p53 protein levels via MDM-2, was detected in 50% of UC-associated adenocarcinomas, 33% of dysplasia lesions and 60% of non-cancerous but inflamed samples. In addition, the density of CpG methylation increased from morphologically normal epithelia to dysplasia and carcinoma. However, the relationship between $p14^{ARF}$ hypermethylation and gene expression was not studied (85). Hypermethylation is a frequent mechanism of *MLH1* silencing in the subset of UC-associated dysplasias and carcinomas with high-level MIN (84). Hypermethylation of the E-cadherin (CDH1) gene has been described in UC-associated cancer. *CDH1* gene promoter methylation was detected in 57% (8/14) of UC-associated cancers. Promoter methylation was associated with decreased E-cadherin protein expression, but methylated and non-methylated cancer cases did not differ in terms of mismatch repair status, differentiation or tumor stage (86). A study by Issa *et al.* noted that *p16* promoter methylation was rare in the morphologically normal colorectal mucosa of UC patients, and no difference from normal controls was observed. However, methylation of *p16* exon 1 was elevated in the regions of morphologically normal mucosa in UC patients with dysplasia, as well as in high-grade dysplasia from these patients, but not in normal mucosa from non-dysplasia containing patients (87). It has been suggested that methylation of *p16* exon 1 is characteristic of 'A-type', or age-related, methylation, whereas methylation of the *p16* promoter occurs in cancers. Increased methylation of other A-type genes, including *ER* and *MYOD*, was also observed in the normal mucosa of patients with dysplasia. The authors suggested the intriguing possibility that this age-related methylation may be due to the elevated rate of cell turnover and oxidative stress characteristic of long-standing UC (87).

Experimental animal models of colitis and colitis-associated carcinogenesis

Spontaneous models of UC

The Cotton-top tamarin develops colitis that clinically, endoscopically and histologically resembles UC in man (88). The colitis in this model responds to medical treatment, and exhibits complications and systemic manifestations similar to those observed in humans, including the development of colorectal cancer in association with colitis (89). However, the practicality of this model is limited by the availability of the animals and the long time-course of disease progression. The chemically induced and genetic models of colonic inflammation do not completely mimic the disease situation found in UC patients (88), but they are more readily available, reproducible and conducive to therapeutic and mechanistic studies.

Genetic models of UC and associated carcinogenesis

Genetic manipulation in rodents has yielded several strains that develop spontaneous intestinal inflammation as well as associated colorectal cancer. Transgenic mice dominant negative for a mutant N-cadherin develop patchy, transmural small bowel inflammation reminiscent of Crohn's disease that is associated with dysplasia and adenoma formation (90). Mice lacking the cell-signaling molecule Ga_{i2} develop left-sided or pan-colitis and ~30% of $Ga_{i2}^{-/-}$ mice develop colorectal adenocarcinoma (91). Transgenic HLA-B27 rats manifest a UC-like disease as well as colorectal adenocarcinoma (92). However, the usefulness of these models in studying colitis-associated carcinogenesis has not been explored.

Il-10 gene knockout mice develop enterocolitis involving the duodenum, the proximal jejunum, and proximal colon (15). Approximately 60% of *Il-10*-deficient mice manifest tumors of the cecum and proximal colon (93). Whereas colorectal tumorigenesis in the $Il\text{-}10^{-/-}$ mouse does not involve *Trp53* or *Apc* alterations, or mismatch repair defects (94), it is associated with over-expression of *Cox-2* in inflammatory cells and myofibroblasts of the tumor stroma (95). *Il-2*-deficient mice develop colitis reminiscent of human UC. Most of these animals succumb to colitis or associated wasting syndrome and anemia within 6 months of age (17). Interestingly, double knockouts of *Il-2* and β_2-microglobulin exhibit less wasting and anemia, and a longer life span. In addition, ~30% of these mice manifest adenocarcinoma of the proximal colon in a setting of pan-colitis with an intermittent disease course (96). The tumors in this model exhibit *Apc* and *Trp53* alterations at high frequencies (100 and 60%, respectively), as well as MIN (96). A third of *Tcr*-deficient mice develop pan-colitis (with no ulceration) as well as inflammation at other sites (18). Colorectal tumors were infrequently observed in *Tcr*β mutant mice, but double mutants harboring *Tcr*β and *Trp53* alterations exhibit colorectal tumor development primarily in the cecum (97).

Chemically induced models

Hapten and acetic acid-induced colitis models. Trinitrobenzene sulfonic acid (TNBS) and dinitrochlorobenzene (DNCB) induce colitis in rodents that displays many characteristics of human IBD. Both chemicals form hapten–protein complexes, leading to T-cell or macrophage responses in the case of TNBS, and a delayed-type hypersensitivity response in the case of DNCB. In contrast, the acetic acid model is based on direct irritation of the colonic epithelium, leading to short-lived ulceration and inflammation (98). These chemically

induced models have been used widely to study the mechanisms and inhibition of colitis. However, colitis-associated carcinogenesis has not been well studied in these models, although TNBS-induced colitis has been shown to promote carcinogen-initiated colorectal tumorigenesis (99).

Carrageenan-induced colitis and colorectal cancer. Colitis induced by sulfated polysaccharides bears histological similarities to human IBD. Degraded carrageenan isolated from red seaweed induces colitis in rodents characterized by mucosal lesions in the cecum and, to a lesser extent, the colon and rectum (100). The mechanism of the carrageenan model is unknown, but germ-free animals are resistant to the induction of colitis, and normal colonic flora is altered, suggesting a bacterial involvement (101,102). Carrageenan-induced colitis in rats has been associated with rectal squamous metaplasia and adenomatous polyps (103) as well as tumor formation (104).

Dextran sulfate sodium (DSS)-induced colitis and colorectal carcinogenesis. DSS-induced colitis. The DSS model has been among the most widely used models of chemically induced colitis and, more recently, colitis-associated colorectal cancer development. DSS is a synthetic, sulfated polysaccharide that induces colitis in rodents that is clinically and histologically reminiscent of human UC. The DSS model in mice is characterized by both acute and chronic UC (105), and may result from altered colonic microflora or macrophage activity (105,106). DSS has also been shown to be directly toxic to crypt cells, and colonic crypt loss may precede the onset of an immune response (107). Susceptibility to DSS-induced UC varies between species and strains (108), indicating a strong interplay within the model between causative factor and background genetics.

DSS-induced colitis-associated colorectal tumorigenesis and mechanistic studies in rodents. Rats administered high molecular weight DSS in the diet for a prolonged period exhibit squamous metaplasia of the rectal mucosa, papilloma of the squamous epithelium, and also adenoma and adenocarcinoma (109). Further studies have shown that rats fed low-dose DSS for an extended period of time (as long as 660 days) also develop squamous cell carcinoma (110). Another group reported colorectal adenocarcinoma in 11 of 13 rats after 6 months of DSS consumption (111). Dysplasia and adenocarcinoma occurred in hamsters maintained for 180 days on low-dose DSS. Seven of eight hamsters in that study developed dysplasia, and four of eight exhibited mucinous, well-differentiated adenocarcinoma (112). However, colitis-associated carcinogenesis in the rat model has not been well studied.

We and others have been studying the colitis-associated carcinogenesis process using the DSS model in mice. Typically, cyclic DSS treatment is used as described by Okayasu *et al.* (105): DSS is administered via the drinking fluid for 3–7 days, followed by water administration for 1–2 weeks to permit healing of the colonic mucosa. The animals are subjected to several such DSS 'cycles' to simulate the course of UC observed in humans, which is characterized by periods of active inflammation (flare-ups) separated by periods of disease inactivity. Using different DSS treatment regimens, Cooper *et al.* analyzed the relationship between the severity of DSS-induced inflammation and colorectal carcinogenesis (113). Higher inflammation scores correlated with a greater occurrence of dysplasia or cancer, and DALMs occurred in settings of mild inflammation whereas flat lesions were associated with colitis of greater severity. DALMs, but not flat lesions, were characterized by β-catenin accumulation in the cytoplasm and nucleus, indicative of *Apc* pathway alteration. Neither type of lesion in the DSS model seems to be associated with *Trp53* alteration (113), not unlike many other murine models of cancer.

The DSS model of chronic UC in mice has also been used to study the mechanistic roles of genes and processes thought to be involved in human UC-associated carcinogenesis. Cyclic DSS treatment promotes colorectal tumorigenesis in the $Apc^{Min/+}$ mouse, with loss of function of the remaining *Apc* allele by mutation (114). This is consistent with human studies, which have indicated that *Apc* alteration is involved in a subset of UC-associated cancers. *Apc* pathway-deficient DALMs occur in the DSS model in the absence of germline *Apc* mutation as well (113). Similarly, cyclic DSS treatment promotes colorectal tumor development initiated by the carcinogen azoxymethane (AOM) (115). Tumorigenesis in the AOM model is known to involve loss of Apc function. *Msh2*-deficient mice, which exhibit the MIN+ phenotype, are more susceptible to adenocarcinoma and dysplasia development when subjected to 3–5 cycles of DSS treatment (116). This model may be useful in studying the subset of UC-associated neoplasms that are MIN-positive.

The role of oxidative stress in DSS-induced, UC-associated carcinogenesis: the effects of iron and N-acetylcysteine. Our laboratory has been studying UC-associated colorectal carcinogenesis and the role of iron supplementation. In our studies, C57BL/6 mice, which are not susceptible to spontaneous colorectal tumor development and moderately sensitive to DSS treatment (108), are subjected to 15 consecutive DSS cycles consisting of 7 days of low-dose DSS treatment followed by 10 days of tap water administration. In our study, invasive colorectal adenocarcinoma and anal squamous cell carcinoma, as well as dysplasia, occurred at rates of ~30% in a setting of moderately severe inflammation (117). The majority of the tumors observed in this model were well-differentiated, mucinous adenocarcinomas, the most commonly observed type of carcinoma observed in UC patients (118).

UC patients frequently experience iron deficiency anemia due to chronic disease and colonic blood loss, and anemia is corrected in these individuals by iron supplementation. In order to assess the role of iron supplementation in the UC-associated carcinogenesis process, we administered diet containing 90 mg iron/kg diet (versus 45 mg/kg iron in the AIN76A diet) to DSS-treated mice. The consumption of diet containing twice the normal level of iron significantly increased colorectal tumor development: the adenocarcinoma incidence increased from ~30% in non-iron supplemented mice to >80% with increased dietary iron. In short-term studies, elevated dietary iron increased the DSS-induced UC index, together with local iron deposition and the expression levels of inducible nitric oxide synthase (iNOS) and nitrotyrosine (117). In a subsequent study, we analyzed the effect of *N*-acetylcysteine (NAC) consumption on cancer development in this murine model. Colorectal tumor incidence and tumor multiplicity were significantly decreased in mice consuming 2000-p.p.m. NAC diet during the water recovery period as compared with positive controls. In addition, consumption of the antioxidant decreased inflammation-driven epithelial cell proliferation, nitrotyrosine staining and iNOS-positive macrophage involvement (119). These results suggest that oxidative stress and inflammation play important roles in the development of colorectal cancer in UC patients. Iron supplementation may contribute to the

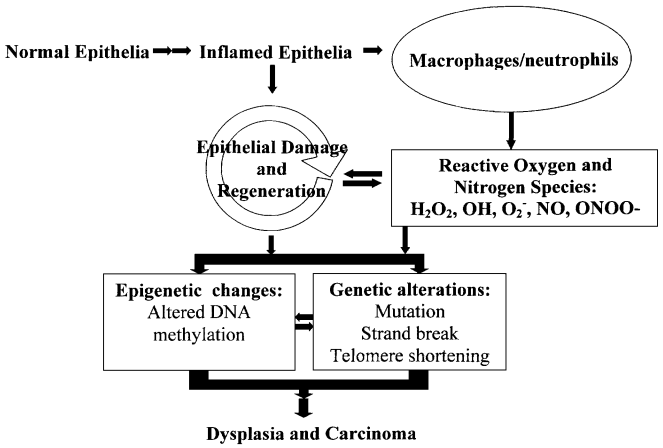

Fig. 1. Proposed roles of oxidative stress and increased cellular turnover in UC-associated carcinogenesis. Reactive oxygen and nitrogen species produced by macrophages and neutrophils in the vicinity of colonic epithelial cells can directly mediate damage to DNA, leading to mutations, DNA strand breaks, and shortening of telomeres, and indirectly affect DNA methylation status. These alterations cause loss of tumor suppressor gene function, gain of oncogene function, and loss of genetic stability. The hyperproliferation of cells in the inflammation-associated damage–regeneration cycle contributes to the fixation of genetic and epigenetic alterations and promotes the development of colorectal dysplasia and carcinoma.

carcinogenesis process by augmenting oxidative damage and inflammation-caused epithelial proliferation. NAC may inhibit these processes by way of antioxidant activities.

Mechanisms of UC-associated colorectal carcinogenesis: oxidative stress and elevated cell turnover

UC and oxidative stress

Oxidative stress and oxidative cellular damage are hallmarks of UC, and probably play key roles in the pathogenesis of this disease and the associated carcinogenesis process. This concept is illustrated in Figure 1. The activities of phagocytic leukocytes are greatly increased in the colons of UC patients (120,121), resulting in enhanced generation of pro-oxidant molecules (122,123). UC manifests deficiencies in antioxidant defences, presumably due to excessive inflammation (124–126). Pharmacological intervention in human UC patients also attests to the role of oxidative stress in this disease. For example, the therapeutic effects of 5-aminosalicylic acids have been attributed, in part, to antioxidant, iron-chelating, and radical scavenging effects (127–130).

Studies using human UC biopsy and colectomy samples have implicated reactive oxygen and nitrogen species (RONS) in the pathogenesis of UC (125,131,132). A significant amount of attention has been paid to the role of nitric oxide (NO). Serum nitrite levels, a measure of nitric oxide synthase (NOS) activity, are increased in active UC patients, and nitrite levels and NOS activity are increased in UC biopsies (133,134). In addition, iNOS activity in inflamed intestinal mucosa is increased and correlates with UC disease activity (135). Epithelial cells in inflamed mucosa express immunodetectable iNOS and nitrotyrosine, demonstrating nitrosative damage of colonic epithelial cells by the NO/superoxide anion reaction product, peroxynitrite (136). Animal models also attest to the involvement of oxidative stress in the pathogenesis of colitis. As in humans, the spontaneous colitis in rhesus macaques is characterized by elevated plasma and urine levels of NO reaction products, increased mucosal iNOS activity and increased mucosal iNOS mRNA levels (137). Radical scavengers, SOD, catalase and NOS inhibitors are therapeutic in chemically induced models of colon inflammation, including the DSS model (138–144). Intra-rectal administration of peroxynitrite induces colonic inflammation in rats (145), and the *iNOS* knockout mouse exhibits attenuated colitis in the DSS model (146). We have shown that dietary iron supplementation exacerbates DSS-induced colitis concomitant with enhanced nitrotyrosine and iNOS expression (117).

Oxidative cellular damage as a potential driving force of UC-associated carcinogenesis

We have found that dietary iron supplementation enhances carcinoma development in the DSS model (117). This effect may be due to an iron-catalyzed increase in oxidative damage. Indeed, iron supplementation in this study was also associated with a rise in nitrotyrosine expression. The role of oxidative damage in this colitis-driven carcinogenesis model was also suggested by the effectiveness of the antioxidant NAC in inhibiting carcinoma development and nitrotyrosine-positive cell number (119). Cellular damage caused by oxidative stress may provide a mechanistic basis for many of the events thought to drive UC-associated carcinogenesis in humans and animal models, including specific gene alterations, genetic instability and aberrant methylation (Figure 1). RONS can covalently modify DNA bases, and failure to remove these lesions leads to base substitutions, deletions and insertions (147,148). RONS have been shown to induce changes in repetitive DNA *in vitro*, and may be a cause of MIN in the inflamed colonic mucosa. The most commonly observed oxidized adduct in human tissues is 8-OHdG, which has been found to cause predominantly G to T transversions *in vitro* (147). Interestingly, this base change is often seen in oncogenes and tumor suppressor genes that have been mutated (149). The products of lipid peroxidation, formed by the reaction of RONS with cell membranes, form etheno- and propano-DNA adducts leading to base transition mutations (150). NO-mediated nitration of 5-methylcytosine and subsequent base deamination leads to C to T transition. NO has also been found to inhibit the function of DNA repair proteins and might thereby act to impair the removal of DNA lesions (151). In inflamed mucosa and cancer samples from UC patients, the *p53* mutation spectrum is dominated by base transitions that may be due to oxidative base modification (149). Other cancer-related genes may also be altered by the action of RONS in the setting of chronic UC.

RONS-mediated DNA breakage and telomere damage may explain the early occurrence of genetic instability during the UC-associated cancer progression. The hydroxyl radical can abstract hydrogen from DNA bases, as well as undergo base addition, leading to apurinic and apyrimidic sites, and strand breaks due to sugar fragmentation (147). Oxidative damage can also mediate double strand DNA breakage if two or more DNA lesions (i.e., single strand breaks) are spaced closely together (152). Repair of these DNA breaks by illegitimate recombination can form mutations and DNA rearrangements, and unrepaired breaks can lead to chromosomal breakage, fragmentation and translocation (153). Telomere shortening is observed in the colonic epithelial cells of UC patients, and telomere length progressively decreases from inflamed epithelia to dysplasia and carcinoma (51,154). This shortening and breakage of the chromosomal ends may cause DNA transloca-

tions, deletions and amplifications. Telomere dysfunction can also lead to loss or gain of whole chromosomes (155,156). It has also been suggested that oxidants can alter DNA methylation patterns. For example, 8-OHdG residues can inhibit the methylation of adjacent cytosines, perhaps leading to DNA hypomethylation and the de-repression of oncogene function (157). It has been suggested that oxidative stress can contribute to hypermethylation of tumor suppressor genes, including *p16* (87), but the mechanistic basis for that effect is not clear and requires further study.

The epithelial damage/restitution cycle and UC-associated carcinogenesis

The DSS model and some of the genetic models of colitis are characterized by an intermittent disease pattern analogous to the flare-up/remission cycle observed in UC patients. The increased cellular turnover that occurs during these 'remission' periods may play an important role in the UC-associated carcinogenesis process. Studies in animal models indicate that inflammation can promote tumor development, such as in the $Apc^{Min/+}$ and AOM models of intestinal tumorigenesis. In both cases, initiated cells are present in the epithelial cell population prior to the onset of colitis. Inflammation-caused proliferation and epithelial regeneration may increase the rate at which additional genetic 'hits' that are needed for further carcinogenic progression occur. For example, the loss of the remaining *Apc* allele in the $Apc^{Min/+}$ mouse. Somatic genetic errors, such as chromosomal non-disjunction events and DNA base mispairs, are normal occurrences during cellular proliferation; but the rates of these anomalies are acceptably low and therefore manageable. The increase in the cell turnover rate that occurs in response to inflammation-caused mucosal damage would be accompanied by a rise in the frequency of replication errors. Enhanced cell turnover would also contribute to the fixation of these changes in the cell population. Genome-wide alterations in DNA methylation occur during normal cellular turnover as well, so-called 'age-related' methylation (87). Elevated cellular turnover may contribute to carcinogenesis by accelerating these global epigenetic changes.

Overall, oxidative stress and elevated cell turnover could work together to produce the genetic and epigenetic changes responsible for cellular transformation in the inflamed colon. Iron over-nutrition may contribute to colorectal carcinogenesis in UC patients by enhancing inflammation and oxidative stress. On the other hand, dietary antioxidants may alleviate oxidative stress and decrease the risk for cancer in UC patients. Further studies in suitable models are needed to confirm these hypotheses and validate potential modalities for the prevention of UC-associated carcinogenesis.

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