1992

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Hypothesis paper

OXYGEN RADICALS IN ULCERATIVE COLITIS

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[Free Radical Biology & Medicine, Vol. 13, pp. 169-181, 1992]

ABSTRACT

This article reviews the pathophysiologic concept that superoxide and hydrogen peroxide, generated by activated leukocytes, together with low-molecular-weight chelate iron derived from fecal sources and from denatured hemoglobin, amplify the inflammatory response and subsequent mucosal damage in patients with active episodes of ulcerative colitis. The putative pathogenic mechanisms reviewed are as follows: (l) Dietary iron is concentrated in fecal material owing to normally limited iron absorption. (2) Mucosal bleeding, characteristic of ulcerative colitis, as well as supplemental oral iron therapy for chronic anemia, further conspire to maintain or elevate mucosal iron concentration in colitis. (3) Fenton chemistry, driven especially by leukocyte-generated superoxide and hydrogen peroxide, leads to formation of hydroxyl radicals. (4) The resultant oxidative stress leads to the extension and propagation of crypt abscesses, either through direct membrane disruption by lipid peroxidation or through generation of secondary toxic oxidants such as chloramines. (5) Chemotactic products of lipid peroxidation, including 4-hydroxynonenal, provide positive feedback to accelerate this inflammatory/oxidative process, leading to acute exacerbations of the disease. (6) Other oxidized products, such as oxidized tryptophan metabolites, created by free radical mechanisms in or near the mucosa, may act as carcinogens or tumor promotors that contribute to the exceedingly high incidence of colon carcinoma in patients suffering from chronic ulcerative colitis. In this way, self-sustaining cycles of oxidant formation may amplify flare-ups of inflammation and mucosal injury in ulcerative colitis. This concept, if proved correct by subsequent research, would provide a rationale for several novel clinical approaches to the management of ulcerative colitis, including use of SOD mimetics, iron chelators, and chain-breaking antioxidants.

Key words: Carcinogenesis, Fenton reaction, Free radicals, Hydroxyl radical, Inflammatory bowel disease, Iron, Ulcerative colitis, Superoxide

Supported by Supported by a Focused Giving Grant from Johnson & Johnson and by Grant HL-42015 from the U.S. Public Health Service, Bethesda, MD.

INTRODUCTION

Ulcerative colitis is an inflammatory bowel disease of unknown cause, characterized by cycles of acute inflammation, ulceration, and bleeding of the colonic mucosa [1-3]. Macroscopically the mucosa in active ulcerative colitis appears hyperemic. It bleeds easily when touched [4]. Microscopically inflammation begins in foci of polymorphonuclear exudate, called crypt abscesses, which develop within microscopic pockets of colonic epithelium (the crypts) and tend to expand and undermine the adjacent normal mucosa to produce interconnected, trench-like ulcerations. These meandering ulcerations tend to surround and isolate islands of residual, intact mucosa, called pseudopolyps, or more correctly, inflammatory polyps [1]. Sometimes adjacent crypt abscesses coalesce to create tunnels covered by mucosal bridges.

Persistent irritation of the colon and loss of normal absorptive function lead to frequent diarrhea, which is the most troublesome symptom of the disease. Bleeding from the ulcerated surface of the colon is chronic and at times severe, as blood vessels are eroded by the ulcerations. Continual blood loss leads to iron deficiency anemia, which may require oral or parenteral iron supplements [2, 4, 5]. The clinical course of the disease includes periods of quiescence or remission, punctuated by acute flare-ups of pain, diarrhea, and bleeding. As years pass, the chances of developing carcinoma of the colon are greatly elevated in patients with ulcerative colitis (roughly 1% per year after diagnosis). The present review explores the potential consequences of the acute and chronic oxidative stress, produced by interactions among the inflammatory cells and extravasated erythrocytes in the submucosa, residual dietary iron, and microflora within the lumen of the colon.

LABORATORY EVIDENCE FOR FREE RADICAL MECHANISMS

Putative pathological chemistry

Although the initial stimulus provoking inflammation of the colon mucosa is not firmly established, there is mounting evidence that the interaction of leukocyte generated superoxide O2- with the relatively high concentrations of low-molecular-weight chelate iron in close proximity to sites of mucosal inflammation plays a key role in the amplification of the inflammatory response and the acceleration of mucosal ulceration during flare-ups of ulcerative colitis. Possible oxidative chemical mechanisms driving exacerbations of the disease include the classical superoxide driven Fenton reaction, auto-oxidation of iron that is reduced by agents other than superoxide, lipid peroxidation by low-molecular-weight ferrous/ferric complexes, chloramine generation, and free-radical-mediated carcinogen activation.

Fenton chemistry

The central toxic mechanism proposed in most free radical theories of disease is the well-known superoxide-driven Fenton reaction,

$$\dot{O}_2^- + A^{-n}Fe^{+++} \rightarrow O_2 + A^{-n}Fe^{++}$$

$$2\dot{O}_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

$$H^+ + A^{-n}Fe^{++} + H_2O_2 \rightarrow H\dot{O} + A^{-n}Fe^{+++} + H_2O,$$

in which iron is shown complexed to a chelator anion A^{-n}. This sequence can provide a ready source of highly reactive hydroxyl radicals ($H\dot{O}$) at physiologic temperatures and pH [6].

Hydroxyl radicals are extremely potent oxidants capable of initiating many deleterious reactions, including lipid peroxidation [7] and carcinogen activation [8]. The effectiveness of the superoxide-driven Fenton reaction in generating measurable quantities of $H\dot{O}$, however, is exquisitely dependent on the nature of the iron chelator, A^{-n}. Common laboratory chelators such as ethylenediaminetetraacetic acid (EDTA) are extremely effective in promoting Fenton chemistry at near neutral pH, while many endogenous biological chelators are not [6].

We have shown that bile pigments, abundantly present in colon contents, can combine with iron to form chelates that are highly effective in the catalysis of $H\dot{O}$ formation by the superoxide-driven Fenton reaction [9]. Accordingly, it is chemically plausible to suppose that if sufficient sources of superoxide and iron can be identified in active colitis, products of free radical reactions initiated by $H\dot{O}$ might participate in the pathogenesis of the disease. In the particular setting of ulcerative colitis, $H\dot{O}$ radicals may be generated by the reaction of leukocyte-derived hydrogen peroxide with ferrous heme iron [10], derived from red blood cells extravasated during mucosal hemorrhage. Human neutrophils stimulated to produce oxygen radicals cause lysis of erythrocytes by a mechanism associated with the oxidation of oxyhemoglobin to methemoglobin, a process that is inhibited by superoxide dismutase (SOD) and catalase [11]. This process can liberate iron. Indeed, the chronically inflamed colon is a particularly well endowed with precursors of the superoxide-driven Fenton reaction--including activated leukocytes that make superoxide, heme iron from microscopic hemorrhage with extravasation and lysis of erythrocytes within the subepithelial space, nonheme iron in fecal contents, and low-molecular-weight chelators of iron that can promote Fenton chemistry--all of which may conspire to subject the colonic mucosa to oxidative stress.

Upon reflection, if one were to list possible disease processes in which the superoxide-driven Fenton were likely to play a significant role, ulcerative colitis would be a likely candidate for the top of the list; for in this condition there is at the surface of the inflamed colonic mucosa a littoral zone, where the greatest known sources of superoxide in biology--activated leukocytes--come in contact with fecal suspensions containing the greatest concentrations of low-molecular-weight

chelate iron normally present within the body. The further addition of heme iron from chronic mucosal bleeding and residual therapeutic iron can only increase the likelihood of iron-mediated oxidative stress in this condition. The interaction of neutrophils and extravasated red cells in colitis may be especially important in that lysosomal enzymes from intact or decaying neutrophils are likely to enhance degradation of hemoglobin iron to low-molecular-weight chelate forms that may further facilitate Fenton chemistry.

Lipid peroxidation by non-Fenton oxidants

In addition to classical Fenton chemistry involving the redox cycling of low-molecular-weight forms of iron, there are intriguing possibilities for generation of non-Fenton oxidants that may be of equal or greater importance in the pathophysiology of ulcerative colitis. Grisham and co-workers have reported that hydrogen peroxide combines with hemoglobin to produce a potent heme-protein-associated radical that peroxidizes polyunsaturated fatty acids and membrane lipids [11]. Products of lipid peroxidation induced by oxo-heme-iron are potent chemoattractants for neutrophils, as are products of lipid peroxidation from the metal-catalyzed Fenton reaction [13, 14]. Aust and co-workers have reported provocative evidence that ferrous/ferric complexes of low-molecular weight chelate iron can initiate lipid peroxidation directly without the intervention of $H\dot{O}$ [15, 16]. These studies, showing that 1:1 combinations of ferrous and ferric iron were most effective initiators of lipid peroxidation, may be especially relevant to the pathophysiology of colitis, owing to the simultaneous presence of both reductants and oxidants of iron in close proximity to the inflamed mucosal surface: the local reductants, including hydrogen sulfide (H_2S) from anerobic bacteria in the lumen and superoxide from activated leukocytes; the local oxidants, including dioxygen diffusing from the hyperemic mucosa and hydrogen peroxide from the dismutation of superoxide.

Chloramine generation

Grisham's research team has also described the toxic effects on colonic mucosa of several chloramines, particularly monochloramine produced from the nonenzymatic reaction of hypochlorous acid (produced by activated neutrophils) and ammonia (produced by gut flora) [17]. Hydrogen peroxide formation in proximity to chloride ions, abundant in intestinal secretions, and certain peroxidases, especially myeloperoxidase (MPO) secreted into the extracellular medium by activated leukocytes, result in the formation of the hypochlorous acid, HOCl [18], a potent oxidant that is the active ingredient in household bleach:

$$H_2O_2 + Cl^- \xrightarrow{MPO} H_2O + HOCl\,.$$

HOC1 is a well-recognized oxidizing and chlorinating agent that reacts with primary amines (RNH_2) to yield N-chloro derivates (chloramines, RNHCl) as follows: HOC1 + $RNH_2 \rightarrow H_2O$ + RNHCl. Chloramines are known to cause cytotoxicity through sulfhydryl oxidation, cytochrome inactivation, chlorination of purine bases on DNA, and degradation of proteins and amino acids [12]. In contrast to primary amines ($R\text{-}NH_3^+$), which are protonated and charged at physiologic pH, chloramines exist primarily in the unchanged form (RNHCl, rather than RNH_2Cl^+) [19].

Thus, the toxicity of these low-molecular weight chlorinated amino compounds is enhanced compared to other oxidants by their lipophilicity, which in turn enhances their rapid absorption and distribution across cell membranes.

Ammonia is produced abundantly by gut flora, notably urea-splitting proteus species [20], and might well enhance formation of monochloramine in the gut, as it combines with leukocyte-derived hydrogen peroxide in patients with ulcerative colitis [21]:

$HOCl + NH_3 \rightarrow H_2O + NH_2Cl$ (monochloramine).

Grisham and co-workers have shown that chloramines in general, and monochloramine in particular, are extremely effective promoters of increased vascular permeability, electrolyte loss, and epithelial cell loss in the rat colon [17]. Among the various oxidants tested, HOCl and NH_2Cl had the most potent toxic effects at physiologically realistic concentrations. In particular, chloramines can directly evoke electrolyte and water loss into the lumen of the intestine, an important potential mechanism for the excessive diarrhea of patients with ulcerative colitis.

Another interesting property of chloramines is their ability to induce lysis of erythrocytes and to impose oxidative stress on their contents, as evidenced by glutathione depletion [19]. This effect has the potential to cause accelerated release of redox active iron species from extravasated red cells that find themselves in proximity of activated neutrophils, exactly as happens in the mucosa and submucosa of the colon in active episodes of ulcerative colitis. Indeed, erythrocyte: neutrophil ratios characteristic of inflammatory infiltrates are exactly those required for maximal chioramine-induced hemolysis [19].

Carcinogen activation

Recently, Eaton [22] and Babbs [9] have proposed that free radical mechanisms may be responsible in some individuals for the initiation or promotion of carcinoma of the colon, a disease that occurs with alarmingly high incidence in patients with ulcerative colitis. In brief, there is considerable evidence from diverse research groups that can be marshaled to support the working hypothesis that (1) dietary iron, liberated from macromolecules during digestion and chelated by bile pigments or amino acids, becomes concentrated in the fecal stream; (2) in the predominantly anerobic environment of the colon a significant fraction of fecal iron is reduced and maintained in the ferrous state; (3) aerobic or microaerophilic fecal microorganisms in the relatively well oxygenated periphery of fecal masses generate superoxide and hydrogen peroxide. Moreover, additional superoxide and hydrogen peroxide may derive from the lipoxygenase activity of epithelial cells themselves or simply from the autooxidation of reduced iron. In turn, (4) these conditions lead to hydroxyl radical formation via the superoxide-driven Fenton reaction, and (5) hydroxyl radicals generated by the superoxide-driven Fenton reaction initiate oxidative chain reactions involving unsaturated lipids and dietary procarcinogens, which transform a fraction of these species into active carcinogens or tumor promotors, capable of inducing neoplasia and/or stimulating cell proliferation in the colonic epithelium.

In terms of specific chemical mechanisms of carcinogen activation, the work of Marnett and coworkers provides the most fascinating possibility. Their research demonstrates in the case of benzo(a)pyrene (BP) that free radical oxidations mimic those produced by the cytochrome P450 system in liver, which is classically known to produce active carcinogens from originally less toxic substrates [23-26]. In particular, the metabolic activation of benzo(a)pyrene hydrodiol to its ultimate carcinogenic form occurs by epoxidation to form the diol epoxide. The resultant diolepoxides are then believed to act as ultimate carcinogens through DNA adduct formation.

Marnett and co-workers, using elegant stereochemical analyses to separate enzymatic from non-enzymatic oxidations, have shown that such epoxidation reactions can and do occur by a free radical mechanism, in which lipid hydroperoxides in the presence of as little as 0.5 μM ferrous iron act as the epoxidizing agents [8, 25-28]. The key step in this novel pathway for activation of polycyclic aromatic hydrocarbons is the nonenzymatic epoxidation of isolated double bonds by lipid peroxyl radicals (LOO*). The mechanism is well precedented in the chemical literature and is known to occur effectively at temperatures between 30 and 60 °C and at PO_2's as low as 10 mmHg [29, 30]. In addition to polycyclic aromatic hydrocarbons like benzo(a)pyrene, aromatic amines may also be oxidized to mutagenic derivatives by peroxyl radicals [23, 31]. In patients with inflammatory bowel disease, lipid traffic through the colon may be specifically increased, owing to the interruption of the enterohepatic recirculation of bile salts and the attendant reduced absorption of fat micelles in the small intestine. Peroxidation of these lipids may be especially important in the formation of carcinogens. Accordingly, such free-radical-mediated activations may well account for the high incidence of colon cancer in patients with chronic ulcerative colitis.

Sources of mucosal superoxide in ulcerative colitis

Activated leukocytes are well-known sources of superoxide radicals and hydrogen peroxide [32, 33]. Over 90% of the oxygen consumed by neutrophils after activation can be accounted for by superoxide secretion [18]. The activation response may be initiated by the binding of mediators to the cell surface or by the process of phagocytosis of particulate material. The oxidants produced by activated neutrophils play a key role in their ability to kill ingested bacteria [32]. However, the oxygen metabolites released from activated neutrophils and macrophages may also be toxic to erythrocytes, endothelial cells, fibroblasts, tumor cells, platelets, and leukocytes themselves [18]. Cumulative amounts of superoxide generated in and around activated neutrophils during a 15-min respiratory burst may be approximately 5 mmol/L, assuming 5 to 20 nmol/10^6 cells/15 min burst [18, 32] within the volume of a sphere 15 μm in diameter. There is good evidence that the oxidase system of the neutrophil is located at least in part on the external surface of the plasma membrane, accounting for the release of substantial amounts of superoxide into the external environment after neutrophil activation [18]. To the extent that superoxide produced by activated leukocytes is liberated from the cell surface into the extracellular space [33], the extraordinarily large amounts of oxidants produced locally are available to react with other extracellular components, including low-molecular-weight chelate iron and other compounds present in intraluminal contents impinging on the inflamed, ulcerated surface of the diseased colon.

Moreover, if, as reported by Vissers and coworkers [34], activated neutrophils adherent to a surface produce oxidants only at the site of attachment, simple calculations lead to a telling insight. Assuming the dimensions of the neutrophil-substrate cleft to be 10 μm in diameter by 0.1 to 1 μm in thickness, as estimated from electron micrographs, then cumulative superoxide generation in the cleft may reach concentrations from 100 to 1000 mmol/L during the respiratory burst. In the case of a tightly adhering neutrophil, the volume of a cleft of radius, r, and thickness, h, is roughly $V = \pi r^2 h = 3.14 \times 25 \times 0.1 = 8\ \mu^3$, and the local cumulative superoxide release into the cleft is

$$\frac{10 \times 10^{-15}\ \text{mole}}{\text{cleft}} \times \frac{\text{cleft}}{8\mu^3} \times \frac{10^{15}\ \mu^3}{\text{L}} = 1.25\ \text{mole/L}.$$

This value represents an extremely large local release of superoxide. Further, the area of contact between the neutrophil and substrate may be inaccessible to antioxidant proteins in the medium such as SOD and catalase, which may be unable to control oxidative stress occurring at this local site.

In this regard, it is important to appreciate that the extracellular space is relatively lacking in antioxidant enzymes, SOD, catalase, and peroxidases, which are concentrated in red blood cells and somatic cells. Since relatively small amounts of SOD are present in extracellular fluids [35, 36]; the release of reactive oxygen species into the interstitium by activated leukocytes could be especially effective in promoting Fenton chemistry in the immediate microenvironment. Another important source of superoxide in colitis may derive simply from the spontaneous auto-oxidation of ferrous iron chelates that have been previously reduced by metabolites of the anerobic subpopulation of fecal flora. Auto-oxidation may occur when reduced iron approaches relatively oxygen-rich mucosal surface, as a result of the spading action of colonic smooth muscle ($Fe^{+2} + O_2 \rightarrow Fe^{+3} + O_2^-$).

Through this mechanism, a predominantly reducing (hypoxic) environment of fecal material is periodically "contaminated" with increased amounts of oxygen present in the reddened, hyperemic surface of the inflamed colon. The relative liquidity of fecal material in colitis, caused by failure of water absorption, and hypermotility of the bowel wall, caused by irritation in mild to moderate cases, may further abet the exposure of reduced iron complexes to the mucosal surface in this pathologic state. The increased amounts of iron, present near the mucosa in colitis derived from degradation of red blood cell hemoglobin or oral iron therapy, would also be available to participate in redox cycling via bacterial reduction and autooxidation.

Other potential sources of superoxide include xanthine oxidase, colonic bacteria themselves, and lipoxygenase activity of the colonic epithelium. The intestine, especially the upper GI tract, is known to be rich in xanthine oxidase [37], an enzyme considered to be a major source of oxygen radicals during reperfusion [38] and during other forms of mucosal injury [39], and which is a classically known source ofsuperoxide radicals [40]. In particular, xanthine oxidase may be preferentially activated in bouts of ulcerative colitis (from the non-oxidant-producing

dehydrogenase form to the oxidant-producing oxidase form) either by proteases released either from inflammatory cells or by lysosomal proteases released during epithelial cell death.

Another ready source of superoxide in the colonic environment is the respiratory activity of bacteria [41-44], notably catalase negative bacteria and E. coli [45]. Finally, there is also the lipoxygenase activity of normal or sloughed colonic epithelial cells [46], which may provide an additional source of superoxide in the immediate microenvironment of colonic epithelial cells. Thus, there are many potential abnormal sources of mucosal superoxide and hydrogen peroxide in patients afflicted with active ulcerative colitis. The most dominant of these is almost certainly activated neutrophils themselves.

Sources of iron in the large bowel

Less well known, but recently reviewed [9], is the ready availability of low-molecular-weight chelate iron in the lower gastrointestinal tract. In normal individuals consuming a typical Western diet, substantial amounts of iron are delivered to the colon, because only a small fraction of dietary iron is absorbed in upper GI tract [47, 48]. Most dietary iron passes into the colon owing to the well-known "mucosal block" mechanism [47, 49], which evidently evolved to protect the body against excessive iron accumulation and resultant hemochromatosis. Accordingly, iron absorption across the epithelium of the small bowel is limited in adult humans by a saturable carrier to about 1 mg of elemental iron per day (maximally, 3 mg/day in anemic states) [50]. Digestion of dietary iron-containing proteins, such as hemoglobin and myoglobin, in the upper GI tract liberates low-molecular-weight iron complexes that are more likely to participate in uncontrolled free radical reactions.

Simple calculations of colonic iron input and output can bracket the relevant range of iron concentrations expected within the lumen of the colon. Consider first a normal individual consuming a diet containing foods of moderate to high iron content (e.g., sirloin steak 3 mg/100 g, spinach 3 mg/100 g, liver 12 mg/100 g, kidney beans 7 mg/100 g) [51], in which 10 mg of iron are consumed per day. (Note that a high protein diet--often synonymous with a high-iron diet --is recommended in ulcerative colitis to mitigate protein wasting from blood and mucous loss associated with chronic, bloody diarrhea.) Normal iron absorption in the upper GI tract is only about 1 mg/d, leaving 9 mg/d residue. Assuming a stool volume in the range of 0.5 to 5 L/d, one would expect fecal iron to be approximately (9 mg Fe)/(0.5 to 5 L) × (1 mmol)/(56 mg) = 32 to 320 μM. Such concentrations are quite sufficient to support near-maximal rates of the superoxide-driven Fenton reaction in vitro [6, 9]. The possible role of dietary iron derived from the consumption of red meat is indirectly supported by epidemiologic data indicating relatively greater incidence of ulcerative colitis in the United States, Britain, and Scandinavia, and relatively lower incidence of the disease in Japan and Latin America [1].

The foregoing calculations, based on normal iron intake in North American diets, do not include several other factors that conspire to raise stool iron in ulcerative colitis. The first is bleeding from the colonic mucosa. The amount of iron contributed by bleeding can be estimated from the observation that these patients often develop iron deficiency anemia [4]. This means that iron loss from bleeding of the ulcerated surface of the colon exceeds maximal iron absorptive

capacity of 3 mg iron/d. Since 1 mL of packed red blood cells contains approximately 1 mg of elemental iron [47], a reasonable estimate of iron loss might be at least 5 mg of elemental heme iron (a value corresponding to blood loss of about 12 mL of whole blood daily, assuming a hematocrit of 40%). During acute flare-ups of the disease, colonic blood loss can be much greater. Thus it is quite possible that the increased input of iron from bleeding into the colonic lumen may more than make up for the increased output associated with diarrhea. Moreover, local concentrations of blood and heme iron in and around mucosal ulcer tracts, where active bleeding is occurring, may be substantially greater than the hypothetical values just calculated by dividing total blood loss by total stool volume. In addition, polyanionic glycoproteins that coat the surfaces of colonic mucosal cells [1] may provide local iron-binding activity, acting like biologic ion exchange resins that attract and hold free iron present in the lumen of the colon.

An intriguing aspect of mucosal bleeding in the pathophysiology of ulcerative colitis is that blood itself may act either a pro-oxidant or antioxidant depending on the conditions. Normal red cells contain abundant catalase and SOD, an enzyme first isolated from bovine blood [52]. To the extent that these protective enzymes are denatured by the harsh conditions of the colon, however, the residual heme iron is quite capable of supporting Fenton chemistry. In the pathophysiological context of ulcerative colitis, the half life of the antioxidant proteins in the colonic environment may be substantially less than the dwell time of the pro-oxidant iron complexes in the neighborhood of the inflamed mucosa. Hydrolytic enzymes from activated neutrophils in crypt abscesses and ulcer tracts may hasten degradation of antioxidant components of extravasated red cells and also hasten the release of redox cyclable iron from complex heme proteins. If the dwell time of fecal material in the colon is sufficiently long (a question yet to be studied), then the pro-oxidant effects would certainly win out. The effect of denatured red cell contents may even be a partial explanation for the often observed greater severity of ulcerative colitis in the distal colon than in the proximal colon [1, 4]. Blood liberated upstream may be rendered a pro-oxidant by the time it reaches the sigmoid colon and rectum.

Another potentially large source of low-molecular weight colonic iron in ulcerative colitis is oral iron therapy, which is recommended in some cases, although often poorly tolerated [5]. If the patient is on daily iron supplementation for treatment of chronic anemia, then stool iron will be sharply elevated. A typical dose of oral ferrous sulfate is one 300-mg tablet, containing 60 mg of elemental iron, three times per day [53]. Since little more than 3 mg will be absorbed, virtually the entire dose winds up in the colon. In such circumstances fecal iron input will be over 10 times normal, tending to drive fecal iron concentration sharply upward, despite any increase in stool volume.

Presence of chelators that support Fenton chemistry

In living tissues, the levels of low-molecular-weight chelate iron, capable of supporting Fenton chemistry, in the plasma or in tissues are extremely low [54, 55] owing to the presence of iron-binding proteins such as transferrin and ferritin. Transferrin, in particular, is able to bind iron tightly and in such a way that it does not participate in the superoxide-driven Fenton reaction 56]. However, such antioxidant, iron-binding proteins, initially present in the diet are very likely to have been degraded and digested in the upper GI tract by the acidity of the stomach contents

and by digestive enzymes such as pepsin and trypsin. These digestive processes make less complex forms of iron available to participate in free radical reactions in the colon.

My colleagues and I have shown that the common bile pigments, which give stool its characteristic brown color, are quite capable of chelating iron in states that readily support the superoxide-driven Fenton reaction [9]. Feces are rich in such bile pigments, including bilirubin, biliverdin, and urobilinogen. These compounds are derived from iron-binding heme pigments during normal breakdown of red blood cells in the liver and spleen and are excreted in the bile [57]. In all such compounds the structure of the opened tetrapyrrole ring is maintained, including the four nitrogens, which may serve as potential iron chelation sites. Although these bile pigments have been proposed recently to function as chain-breaking antioxidants [58, 59]; they are also excellent iron chelators of the type that not only keep iron soluble at near neutral pH but also promote, rather than inhibit, Fenton chemistry [9]. Heme itself, derived from enzymatic digestion of hemoglobin from mucosal hemorrhage, can also serve as an iron chelator that can support the superoxide-driven Fenton reaction [10].

Other potential low-molecular-weight chelators of iron that may be abundant in the GI tract are the dicarboxylic amino acids, aspartate and glutamate. These amino acids may be liberated preferentially by the action of proteolytic enzymes from dead or dying neutrophils in the inflamed, ulcerated colon mucosa. The action of neutrophil derived proteases upon mucus covering colonic mucosal cells would release more iron-binding amino acids precisely during the times of acute flare-ups of the disease, providing yet another mechanism for positive feedback. Deighton and Hider [60] have isolated oxotriiron complexes of these amino acids (e.g., $Fe_3O[glu/asp]_6$) from rat liver, in which the ratio of glutamate to aspartate is typically 7:2. Such dicarboxylic acid complexes with iron might also support Fenton chemistry within the intestinal tract.

Sufficiency of oxygen

Measurements of oxygen tension at the surface of the normal colon reveal partial pressures of 30 to 40 mm Hg [61], which have been shown both theoretically and experimentally [62] to permit half maximal rates of lipid peroxidation. Thus, although the surface oxygen tension of colonic mucosa is less than that of arterial blood, it is adequate to support lipid peroxidation. Moreover, the increased local blood flow within the hyperemic, inflamed mucosa would certainly tend to enhance oxygen delivery to the diseased mucosa of patients with ulcerative colitis. Clinically, such hyperemia is evident as diffuse reddening at the time of endoscopic diagnosis [4]. Mucosal hyperemia--in conjunction with smooth muscle spasm to provide mixing-- may be especially important in supporting auto-oxidation mechanisms, namely

$$Fe^{+3} \xrightarrow{\text{reductants}} Fe^{+3}$$

$$Fe^{+2} \xrightarrow{O_2} Fe^{+3} + O_2^{\cdot-}$$

In this regard, the experimental studies of Hauser and co-workers in Los Angeles provide a fascinating paradox [61]. They studied surface oxygen tension of the colonic mucosa in normal rabbits and those with experimentally induced colitis, using a modified Clark PO_2 electrode, adapted from those used for measuring conjunctival PO_2. They found normal colonic surface oxygen tension was about 36 mmHg. Interestingly, however, animals developing mild colitis, manifest by erythema of the distal colon and the microscopic appearance of inflammatory cells in the lamina propria, exhibited mucosal surface PO_2's of only 10 to 15 mmHg. In regions of severe colitis, the mucosal surface PO_2 was near 5 mm Hg. At first this result is surprising, since visible erythema of the bowel implies increased blood flow associated with the inflammatory state. Yet in the context of the free radical hypothesis the findings are explainable, if oxygen is being rapidly converted to superoxide and hydrogen peroxide through the respiratory burst activity of neutrophils. In this way the free radical hypothesis is able to explain the apparent paradox of simultaneous hyperemia and low surface PO_2 in experimental colitis.

Proposed consequences of oxidative stress in ulcerative colitis

Direct effects of oxidative stress in mucosal ulcer formation

Oxidative stress may be a direct and proximal cause of mucosal ulceration in flare-ups of ulcerative colitis. At the opposite end of the gastrointestinal tract there is evidence for the role of oxygen radicals in the formation of gastric ulcers [39, 63], which can be suppressed in some experimental models by intravenous administration of SOD. Perhaps similar oxidant-dependent mechanisms might be operative in ulceration of the colon as well. Certainly the biological oxidants involved in Fenton chemistry are capable of altering structural components of tissue, including proteins, carbohydrates, lipids, and nucleic acids [64, 65]. One possible model for such direct effects of free radical reactions in extension of crypt abscesses involves hydrogen peroxide generated from either spontaneous or SOD catalyzed dismutation of superoxide derived from activated neutrophils within the cul de sac of the crypt abscess. Assuming that **extracellular** catalase levels are relatively insufficient, a stream of hydrogen-peroxide- rich mucus and exudate, created within the abscess, would emerge from the crypt orifice to encounter ferrous iron previously reduced in the relatively hypoxic environment of the colonic lumen.

This efflux of hydrogen peroxide from the crypt orifice would occur whenever H_2O_2 generation exceeds the catalase activity of mucus and bacteria within the crypt. As the two steams of material mix near the crypt opening, Fenton's reaction is likely to take place, leading to the creation of toxic oxidants that induce first denaturation of the local mucus barrier, followed by lipid peroxidation within the nearby surface mucosa. This oxidative stress could lead to cell death and epithelial sloughing. The resulting epithelial cell loss may produce a crater that enlarges and deepens as the mixing zone is displaced more toward the bottom of the crypt. Lateral extension could then occur by the same process, producing ulcer tracts separating pseudopolyps. The linear patterns of ulcerations might even correspond to the distribution of underlying blood vessels where oxygen tension is highest. Another possible explanation for the linear patterns of the ulcers is that the flow of colon contents toward the anus carries a streak of activated neutrophils and hydrogen peroxide "downstream" of the crypt opening, producing a

streak-like pattern of oxidative injury. In this way ulceration might be initiated by Fenton chemistry as H_2O_2 escapes from crypt openings to react with fecal ferrous iron at the mucosal surface.

A related possibility is that superoxide is produced locally by neutrophils exuding from the crypt orifice and acts as the principal reductant of iron after it has been oxidized by hydrogen peroxide. This scheme would provide a mechanism for immediate, local redox cycling of iron and obviate the need for a continual fresh supply of ferrous iron from the colon lumen or the need for hydrogen peroxide to escape traces of catalase in its journey from the bottom of the crypt to its orifice. To the extent that SOD itself or SOD mimetics protect against inflammation in inflammatory bowel disease [66, 67] (see below), and to the extent that Fenton chemistry is involved, the action of superoxide as a reductant of iron must be important. Because of the limited biological life span of superoxide radicals, such results would argue for substantial superoxide production directly at the mucosal surface near the crypt orifice. Further, degradation of the surface glycocalyx by neutrophil-derived proteases would make the colonic mucosal surface more vulnerable to lipid peroxidation induced by extracellular oxidants. For these reasons, it is reasonable to suggest that direct toxicity of reactive oxygen species to epithelial cell membranes may be important in the extension of crypt abscesses and linear ulcerations.

Indirect effects of oxidative stress (chemotaxis)

The chemotactic effects of the products of lipid peroxidation may provide an important pathway for positive feedback, the likes of which is needed to explain the explosive nature of sudden exacerbations of clinical ulcerative colitis. When human plasma is incubated with the xanthine-xanthine oxidase system as a source of superoxide, a potent chemotactic factor is generated [18], which appears to be a heat-labile lipid bound to albumin, active at a concentration of 3 ng/mL and distinct from arachadonic acid. In the setting of acute colitis, the effects of neutrophil-derived oxidants in the lamina propria of the large intestine may be especially severe, owing to the relative scarcity of antioxidant enzymes (SOD and catalase) in this extracellular microenvironment [35, 36]. Such chemotactic factors derived from oxygen metabolites released by phagocytic cells may function as a positive feedback mechanism to potentiate the inflammatory response.

According to the free radical hypothesis, the initial crypt abscesses, formed in response to the underlying autoimmune vasculitis or other as yet unidentified inciting agents, are expanded and enlarged by oxidative stress resulting from the combined action of activated neutrophils and fecal iron. One of the most interesting products of free-radical-mediated lipid peroxidation is 4-hydroxynonenal (4-HNE), which has been shown to be an extremely potent chemotactic agent, effective at concentrations as low as 10^{-12} to 10^{-14} M [13, 14, 68]. Quite possibly lipid peroxidation within the mucosa produces chemo-attractants like 4-HNE at the same time that direct effects of oxidative stress lead to physical denudation, erosion, and local bleeding. The required unsaturated lipids may derive from dietary sources, from the turnover of injured epithelial cells, or, as suggested by at least one report [69], from synthesis by fecal microorganisms. As a consequence, more neutrophils and iron are provided to perpetuate the cycle of inflammation, ulceration, and bleeding.

Antioxidants in experimental colitis

Several studies have been reported indicating the partial efficacy of the antioxidant enzymes SOD and catalase in attenuating experimental colitis in animal models. Fretland and co-workers [70] studied acetic-acid-induced colitis in mice and guinea pigs. They tested three types of SOD in an attempt to modulate colonic inflammation. Whenever recombinant human SOD or bovine SOD was given as an enema both 10 min before and 30 min after colitis induction, there were significant reductions in biochemical and microscopic indices of inflammation. Interestingly, manganese SOD was inactive in this paradigm.

Burakoffand co-workers [71] studied the effects of recombinant human SOD (1 mg/kg, i.v.) in rabbits either 1 h before or 1 h after induction of colitis with trinitrobenzenesulfonic acid. SOD administered either as a pretreatment or as a post-treatment sharply inhibited tissue eicosanoid accumulation and significantly reduced the histological inflammation index in treated animals compared to controls. Haydek and co-workers [72] evaluated the effect of catalase on the severity of colitis induced with acetic acid or mitomycin-C in rats. Histological examination of the colon after 4 d revealed about a 50% reduction in numerical indices of inflammation. Each of these studies lends experimental support to the role of biological oxidants in the pathogenesis of colitis and the potential therapeutic effects of antioxidant enzymes in inflammatory bowel disease.

CLINICAL EVIDENCE FOR FREE RADICAL MECHANISMS

Several lines of clinical evidence indirectly support the plausibility of oxidative mechanisms in ulcerative colitis. These include a number of miscellaneous clinical observations, recent work suggesting that the most effective drug in the management of ulcerative colitis may actually work as an antioxidant, and the results of a recent uncontrolled clinical trial of super oxide dismutase in the treatment of inflammatory bowel disease.

Circumstantial clinical evidence

Clinical observations about the natural history of the disease provide intriguing hints that are at least consistent with oxidative mechanisms. The time course of the disease--characterized by near normal periods of quiescence followed by the sudden onset of bleeding and diarrhea--fits with the notion of an explosive, positive feedback mechanism for amplification of the colitis, similar to that proposed. The ability of patients typically to remember the exact date of the onset of such alarming symptoms [3] is testimony to the suddenness and severity of flare-ups of the disease.

The variable and unpredictable timing of flares is also consistent with oxidative mechanisms, which are highly dependent on the balance of oxidative stress vs. antioxidant defenses. In the case of classical chain breaking (Vitamin E-like) antioxidants in particular, high rates of chain propagation occur only when antioxidant has been consumed [29,73]. To the extent that dietary iron and antioxidant consumption vary independently, flares may tend to occur at seemingly

unpredictable intervals when dietary iron intake is especially high and dietary antioxidant intake is especially low.

The role of psychic stress in ulcerative colitis has been much debated [2, 3, 5]. Although there is no evidence that the disease is caused by stress, there is the strong clinical impression that the course of the disease is influenced by stress [2, 3]. One known hematologic response to stress associated with enhanced release of adrenocorticotrophic hormone (ACTH) and epinephrine is an increase in circulating neutrophils [74]. These free radical sources may provide a link between the onset of stress and ulcerative colitis flares that is understandable in the context of an oxidative stress mechanism.

Another clinical observation that provides a clue to pathophysiology is that the presence of crypt abscesses and mucin depletion are correlated with clinical disease activity [3]. This observation has led to the mucin defect theory of the genesis of ulcerative colitis. Mucin is likely to act as a natural protective barrier, which normally protects the bowel epithelium from potentially noxious intraluminal agents. Kim and Byrd [75] have proposed that defects in mucin synthesis or enhanced mucin degradation may be important in the pathophysiology of ulcerative colitis. Grisham and co-workers have recently shown that hydroxyl radical degrades and depolymerizes purified mucin, as measured by decreases in viscosity [76]. Thus, if one considers mucin depletion as the result of increased oxidative stress, perhaps along the biochemical lines studied by Baker and co-workers [77] for the degradation of articular cartilage proteoglycan, the mucin defect theory and the oxygen radical theory become unified. Oxidants like $H\dot{O}$ or hypochlorite from activated neutrophils cause both mucin degradation and mucosal injury. A further positive feedback loop may be involved, because mucin depletion would tend to expose the underlying mucosa to chemotactic substances present in feces, such as N-formyl-methionylleucyl-phenylalanine (FMLP), a tripeptide known to be released from E. coli during replication [12]. Indeed, intrarectal administration of 1 mM FMLP in animals with a normal mucin barrier has been reported as a model of colitis [78]. In this way, oxidative stress may beget mucin degradation, which begets further oxidative stress from activated neutrophils, etc. The particularly high incidence of carcinoma of the colon in patients with persistent ulcerative colitis also fits with oxidative theories of colon carcinogenesis.

One version of this theory, already alluded to [9], posits that oxygen radical reactions in the colon activate dietary procarcinogens or tumor promoters. Because of the strong association of colon cancer with ulcerative colitis, free radical theories of the two diseases are mutually reinforcing. Perhaps the same underlying chemistry, involving iron and oxygen radicals, may be responsible for both the inflammatory and the neoplastic complications of ulcerative colitis.

Effectiveness of Azulfadine (sulfasalazine), which may act as a non-absorbable, chain-breaking antioxidant

Another major clue to oxidative mechanisms in the pathophysiology of ulcerative colitis comes from recent understanding of the mechanism of action of sulfasalazine. Sulfasalazine is the most commonly prescribed drug for the management of ulcerative colitis. The compound consists of one molecule of sulfapyridine linked by an azo bond to 5-aminosalicylate. Typically 2 to 4 g/d

are taken by ulcerative colitis patients [79]. Up to 8 g/d may be prescribed [3]. Sulfasalazine is poorly absorbed in the upper GI tract and in the colon and consequently reaches very high concentrations in the feces [80]. A portion of that which is absorbed reenters the intestine with the bile. After passing unmodified through the upper GI tract into the colon, sulfasalazine is metabolized by enteric bacteria to yield 5-aminosalicylic acid and sulfapyridine.

Although sulfasalazine was originally developed as an antibiotic, it is now rather well accepted that the drug does not alter the microflora of the intestine and works by some property other than its antibacterial activity [79]. Despite its high concentrations in the gut, it is not effective in treatment of bacillary dysentery [81]. It is now well accepted that 5-aminosalicylate is the active moiety of sulfasalazine [79]. In the present context of the oxidative stress hypothesis, it is intriguing to consider that sulfasalazine may work as an antioxidant.

Salicylates are extremely effective hydroxyl radical scavengers [82] with rate constants, $k = 2 \times 10^{10}\ M^{-1}sec^{-1}$, that approach diffusion limited values. Because such large quantities of sulfasalazine are given (and required), the concentrations achieved approach those necessary for efficient scavenging of $H\dot{O}$ [83]. In addition to acting as a phenolic antioxidant, there is also the intriguing possibility that 5-ASA may also function as an iron chelator that inhibits Fenton chemistry. As recently described by Grisham [84], 5- ASA forms a purple chelate with ferrous iron, in which form the iron is unable to oxidize deoxyribose. Interestingly, the N-acetylation of 5-ASA reduced its antioxidant effectiveness in the deoxyribose assay [84].

In yet other variations of this theme, Dallegri and co-workers in Italy have found evidence that 5-ASA is an especially potent scavenger of neutrophil-derived hypochlorous acid [85] and Allgayer and co-workers in Germany have reported that 5-ASA is an effective intracellular and extracellular scavenger of superoxide [86]. These observations suggest that one of the most effective specific drugs in the empirical clinical treatment of ulcerative colitis happens to be a multifaceted antioxidant that is selectively concentrated in the colon and exerts its protective effect by chelating iron and by scavenging biological oxidants.

Superoxide dismutase in the treatment of Crohn's disease

A closely related inflammatory bowel disease that provides a clue to the free radical nature of ulcerative colitis is regional enteritis or Crohn's disease, a chronic inflammatory condition of the small intestine that can also involve other regions of the GI tract from esophagus to rectum as well. Ulcerative colitis and Crohn's disease are often considered together in textbooks because of their overlapping clinical and pathological features. When Crohn's disease involves the colon, it is sometimes difficult to distinguish initially from ulcerative colitis, in which cases the term intermediate colitis may be used [3]. In 1989 Emerit and co-workers reported results of an 8-year Phase II clinical trial, in which intermittent doses of copper-zinc superoxide dismutase, injected intramuscularly, were tried in the treatment of Crohn's disease and the dose regimen was progressively modified to optimize results [66, 67]. In this study, 19 of 26 patients showed good short-term responses to SOD treatment, as measured by a standard Crohn's disease activity index, based on patients' ratings of diarrhea, abdominal pain, well-being, as well as objective findings of an abdominal mass or anemia. Although the chronic, relapsing nature of Crohn's

disease makes it difficult to tell which patients would have spontaneously improved without SOD therapy, these encouraging results are consistent with the view that the symptoms and signs of inflammatory bowel disease may be mediated in part by toxic oxidants.

SUMMARY AND IMPLICATIONS

Ulcerative colitis is a significant disease of otherwise healthy young adults in the prime of life. The incidence of the disease, in high-risk areas, about five new cases per 100,000 population per year, has been increasing over the past few decades [1]. The proposed oxidative mechanisms outlined in the present review for the sudden amplification of inflammation, ulceration, and bleeding that characterize acute flare-ups of the disease may be summarized as follows:

1. Dietary iron is concentrated in fecal material owing to normally limited iron absorption; this fecal iron is present in low-molecular-weight forms, capable of redox cycling.

2. Mucosal bleeding, characteristic of ulcerative colitis, as well as supplemental oral iron therapy for chronic anemia, conspire to further elevate fecal iron (or at least compensate for the dilutional effects of increased stool volume in colitis).

3. Fenton chemistry, driven by leukocyte-generated superoxide and hydrogen peroxide, leads to formation of $H\dot{O}$; non-Fenton oxidants are produced by the combination of heme iron from extravasated red cells and hydrogen peroxide produced by accumulated neutrophils; monochloramine formation results from the nonenzymatic reaction of leukocyte-generated hypochlorous acid with ammonia generated by colon bacteria.

4. These processes combine to produce an extreme degree of oxidative stress, which is important in altering the permeability of the intestine and in the extension and propagation of crypt abscesses, through denaturation of surface mucous or through lipid peroxidation of epithelial cell membranes.

5. Chemotactic products of lipid peroxidation, including 4-hydroxynonenal, provide positive feedback to accelerate and perpetuate the inflammatory process, leading to acute exacerbations of the disease.

6. Other oxidized products, such as oxidized tryptophan metabolites, created by oxidative mechanisms in or near the mucosa [87] may act as carcinogens or tumor promotors that contribute to the high incidence of cancer in patients suffering from ulcerative colitis.

Although these mechanisms do not address the primary cause of ulcerative colitis, which remains unknown [88]; they do propose specific chemical and physiological explanations for sudden exacerbations of the disease that make life miserable for affected patients, and in extreme cases of toxic megacolon, may even lead to death.

The putative free radical mechanisms just reviewed, if proved correct even in part, could provide significant insights for improved management of patients with ulcerative colitis, not only to prevent acute flares of pain, bleeding, and diarrhea, but also to prevent late development of invasive cancer. A number of theoretical possibilities for drug interventions come to mind that are specifically related to attenuating oxidative stress. These include blocking superoxide formation by phagocytes; scavenging superoxide before it can react with iron; binding iron so it does not redox cycle; and scavenging $H\dot{O}$ or HOCl. In addition, certain preventive measures involving diet and iron therapy are suggested by the free radical hypothesis. These potential improvements in management include oral SOD mimetics, oral iron chelators, oral chain-breaking antioxidants (Vitamin E, BHT, β-carotene), colonization of the colon with antioxidant-producing bacteria, control of dietary iron, lipids and fiber, and reassessment of the virtue of parenteral, rather than oral iron therapy.

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