Oxidative Stress in Digestive Disease
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Neutrophil-Dependent Oxidative Stress in Ulcerative Colitis

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Summary **Neutrophil accumulation within epithelial crypts and in the intestinal mucosa directly correlates with clinical disease activity and epithelial injury in ulcerative colitis (UC). Current advances have defined the mechanisms by which neutrophils are activated or migrate across mucosal epithelia. A better understanding of this process will likely provide new insights into novel treatment strategies for UC. Especially, activated neutrophils produce reactive oxygen and nitrogen species within intestinal mucosa, which induce oxidative stress. In clinically, we have succeeded to develop a novel granulocytes adsorptive apheresis therapy for UC. In this article, we discuss current advances to define the role of neutrophils-dependent oxidative stress in UC.**

Key Words**: dextran sodium sulfate, 4-hydroxy-2-nonenal, leukocytapheresis, neutrophill, ulcerative colitis**

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Introduction

The incidence of ulcerative colitis (UC) is increasing year by year in Japan. Although it is clear that genetic, environmental, immunological factors affect the pathophysiology of UC, the direct cause of this disease is still unknown. One of the most prominent histological features observed in UC is infiltration of neutrophils into the inflamed mucosa. Disease activity in UC is linked to an influx of neutrophils into the mucosa and subsequently into the

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intestinal lumen, resulting in the formation of so-called crypt abscesses. In addition, circulating activated neutrophils, a major source of inflammatory cytokines, are elevated with increased survival time in active UC. More importantly in the clinical field, several studies showed that granurocytes/monocytes adsorptive apheresis therapy could induce the remission stage especially in patients with steroid-refractory and steroid-dependent moderate-to-severe UC. In this review article, we would like to offer an up-date data on the role of leukocytes, especially neutrophils, in UC.

Leukocyte and Vascular Endothelial Cell Interaction

The sequence of events in the extravasation of neutrophils from the vascular lumen to the extravascular space is divided into 1) margination and rolling, 2) adhesion and transmigration, and 3) migration in interstitial mucosal tissues towards chemotactic stimulants (Fig. 1), which are regulated by the interaction of adhesion molecules located on the surface of neutrophils and endothelial cells [*1*, *2*]. With stimuli, such as various cytokines and inflammatory mediators, neutrophils roll slowly on endothelial cells through interactions between L-selectin and carbohydrate antigen on neutrophils, and P- and E-selectin on endothelial cells. Eventually, the neutrophils adhere strongly to endothelial cells via CD11/CD18 glycoproteins and endothelial adhesion molecules of immunoglobulin superfamily, including the intercellular adhesion molecule 1 (ICAM-1) and the vascular cell adhesion molecule 1 (VCAM-1). Their expression is stimulated by molecule such as inflammatory cytokines and lipopolysaccharide (LPS). After firmly binding to the endothelial surface, the neutrophils transmigrate between cells along the intercellular junction. Platelet endothelial cell adhesion molecule 1 (PECAM-1), a cell-cell adhesion molecule, is a likely candidate for mediating this process. After passing the endothelial junctions, leukocytes are able to cross the basement membrane by focally degrading it with secreted collagenases. After extravasation, neutrophils emigrate towards the site of intestinal mucosal injury along a chemical gradient of chemotaxis. In UC, exogenous and endogenous substances are able to act as chemotactic agents for neutrophils including 1) cytokines especially interleukin-8 (IL-8), 2) soluble bacterial products, particularly peptides with N-formyl-methionine termini, 3) components of complement system, and 4) products of the lipoxygenase pathway of arachidonic acid metabolism, particularly leukotriene B_4. Current data suggests that Toll-like receptors (TLR), which recognize specific pathogen-associated molecular patterns (PAMPs), are differentially expressed on both leukocytes and mucosal epithelial cells while serving to modulate leukocyte-epithelial interactions. Exposure of epithelial TLRs to microbial ligands has been shown to result in transcriptional upregulation of inflammatory mediators whereas ligation of leukocyte TLRs modulates specific anti-microbial responses. A better understanding of these events will hopefully provide new insights into the mechanisms of epithelial responses to microorganisms and ideas for therapies aimed at inhibiting the deleterious consequences of mucosal inflammation in UC.

Neutrophil-Dependent Mucosal Injury

Neutrophils contain two major granule population, primary (azunophil) and secondary (specific) granules, which are

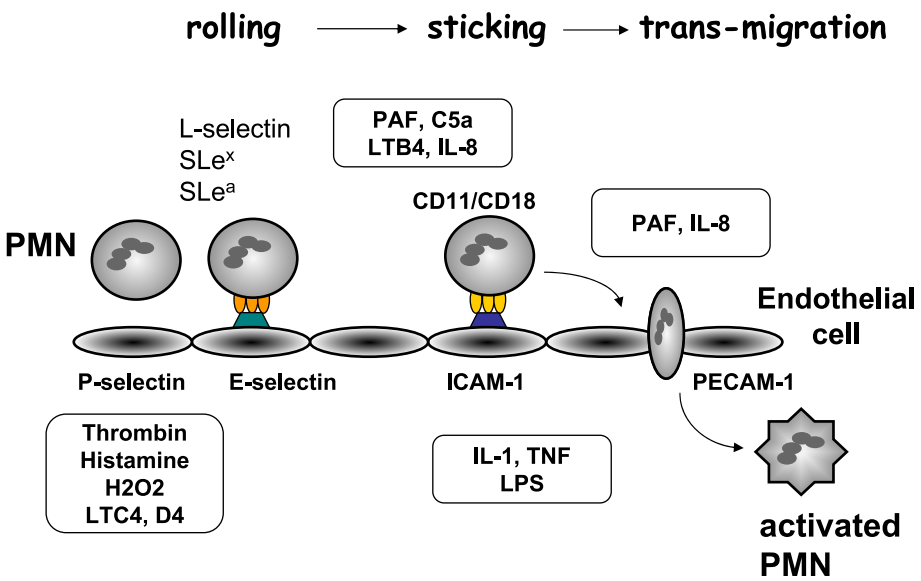

Fig. 1. Neutrophil-endothelial interaction mediated by adhesion molecules and chemical mediators

formed at different stages of neutrophil maturation. Primary granules contain, as their main constituents, several proteolytic enzymes and a wide range of bactericidal proteins including cathepsin G, elastase, myeloperoxidase (MPO), and lysozyme. The secondary granules contain a wide variety of different components, including lactoferrin, lysozyme, collagenase, and lipocalins. In activated neutrophils, NADPH oxidase in cell membranes becomes activated, and an electron transfer takes place from NADPH in cells to oxygen inside and outside cells, and the oxygen that received electrons becomes superoxide radicals ($O_2^{-\cdot}$), which is rapidly converted to hydrogen peroxides (H_2O_2) by spontaneous dismutation or enzymatic superoxide dismutase (SOD), and hydroxyl radicals ($^{\cdot}OH$), which are formed nonenzymatically in the presence of Fe^{2+} as a secondary reaction [*3*, *4*].

Reactive oxygen species (ROS) are highly reactive. When they are generated close to cell membranes, possibly by intestinal epithelial cells, they induce oxidative stress and oxidized membrane phospholipids (lipid peroxidation), which may continue in a form of a chain reaction. Biomembranes contain large amounts of polyunsaturated fatty acids (PUFAs) in their phospholipids. PUFAs contain two or more carbon-double bonds within their structure. This makes them susceptible to oxidative damage by free radical attack, which is a direct cell injury induced by oxidative stress. PUFAs of cell membranes are degraded by lipid peroxidation with subsequent disruption of membrane integrity, suggesting that lipid peroxidation mediated by oxygen radicals is an important cause of damage and destruction of cell membranes. We have shown in a model of trinitrobenzene sulfonic acid (TNBS)-induced colitis that thiobarbituric (TBA)-reactive substances, an index of lipid peroxidation, significantly accumulate in the colonic mucosa [*5*], and treatment with SOD led to an improvement in the colitis score. We have also demonstrated that TBA-reactive substances are significantly increased in the colonic mucosa after dextran sodium sulfate (DSS) administration, and that this increase is significantly inhibited by treatment with Mn-SOD [*6*] and a synthetic vitamin E analogue [*7*] in the same DSS colitis mouse model; this suggests that induction of lipid peroxidation is an early critical event in this experimental inflammatory bowel disease model.

In addition to direct products derived from activated neutrophils, the secondary products induced by oxidative stress may play a role in the development of intestinal inflammation. Experimental and clinical evidence coming from different laboratories suggest that 4-hydroxy-2-nonenal (HNE, Fig. 2), a product during lipid peroxidation, can act as bioactive molecules in either physiological or pathological conditions. HNE can affect and modulate, at very low and nontoxic concentrations, several cell functions, including signal transduction, gene expression, cell proliferation, and more generally, the response of the target cells [*8*–*10*]. Recent our preliminary study has also demonstrated HNE-modified proteins in colonic mucosal obtained from the patients with UC using mono- and poly-clonal antibodies against HNE. The interaction of HNE with a variety of kinases variously involved in cell signaling associated with inflammation is now a matter of active investigation. In

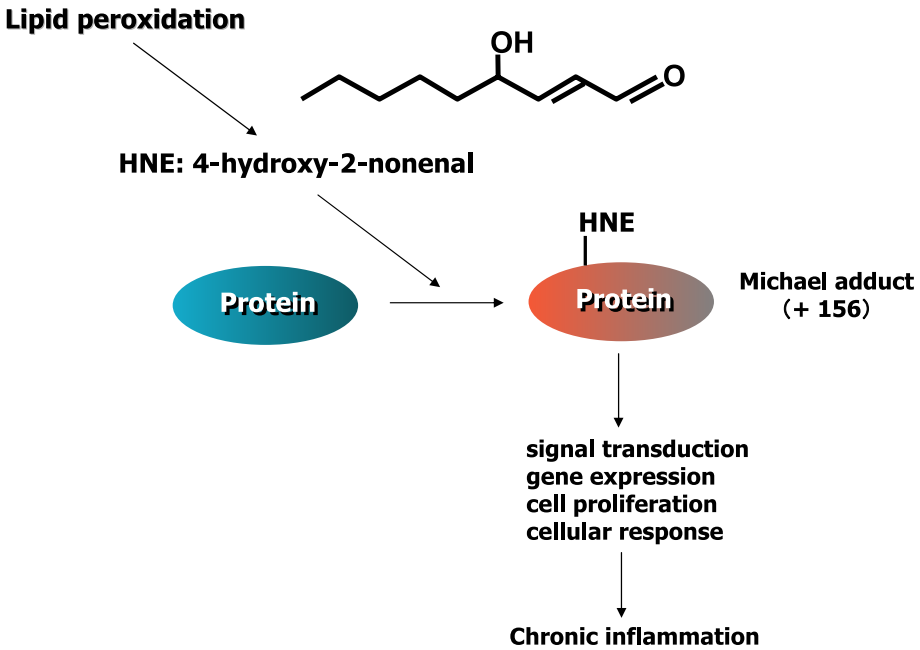

Fig. 2. 4-Hydroxyl-2-nonenal (HNE) protein adducts as a second messenger

particular, findings with regard to the effect of HNE on different components of the protein kinase C family and the mitogen-activated protein kinase complex already provide reliable indications of a potential role of this aldehyde as a cell signal messenger. Such a role appears further supported by the clear-cut evidence of up-regulation of receptor tyrosine kinases and down-regulation of the nuclear factor κB system, produced by HNE concentrations actually detectable in pathophysiology of intestinal inflammation. Recent advance in proteomics technology makes it possible to determine specific targets modified by oxidative stress, including HNE. This modification of proteins may be a biomarker for evaluation of disease activity or therapeutic efficacy.

Role of Neutrophil in Experimental Models of Colitis

Oral administration of DSS induces colitis in animals, which histologically mimics human UC [*11*]. It has been reported that the direct action of DSS on colonic epithelial cells, macrophages, and intestinal microflora are likely to be involved in the pathogenesis of this colitis. In addition, recent reports have hypothesized that neutrophil-mediated inflammation is involved in the development of DSS-induced colonic mucosal injury. Several lines of evidence support this hypothesis: 1) colonic mucosal endothelial ICAM-1 expression is enhanced at an early stage in the inflammatory cascade of DSS-induced colitis [*12*] and in patients with UC [*13*], and immunoneutralization of ICAM-1 significantly attenuated colonic mucosal injury and neutrophil accumulation [*14*], 2) numerous neutrophils accumulates in the DSS-treated colonic mucosa, and selective depletion of neutrophils by a monoclonal antibody reduces DSS-induced colitis [*15*], and 3) scavengers or inhibitors of the neutrophil-derived products also inhibit this type of colitis in animals [*6*, *16*].

Natsui *et al.* [*15*] firstly demonstrated that intraperitoneal injections of RP-3, a monoclonal antibody capable of selectively depleting neutrophils, significantly suppressed bleeding, tissue myeloperoxidase activity, chemiluminescence production and erosion formation in DSS-induced colitis in rats. We have also shown that MPO activity, an index of tissue-associated neutrophil accumulation, significantly increases in the colonic mucosa after DSS administration, and this increase is significantly inhibited by treatment with MnSOD (Fig. 3). These results indicate that the inhibition of neutrophil accumulation by MnSOD may be one of the protective factors decreasing DSS-induced colonic mucosal injury. The inhibition of neutrophil accumulation by SOD was first reported by Grisham *et al.* [*17*] using an *in vivo* model of intestinal ischemia. Recent studies have confirmed

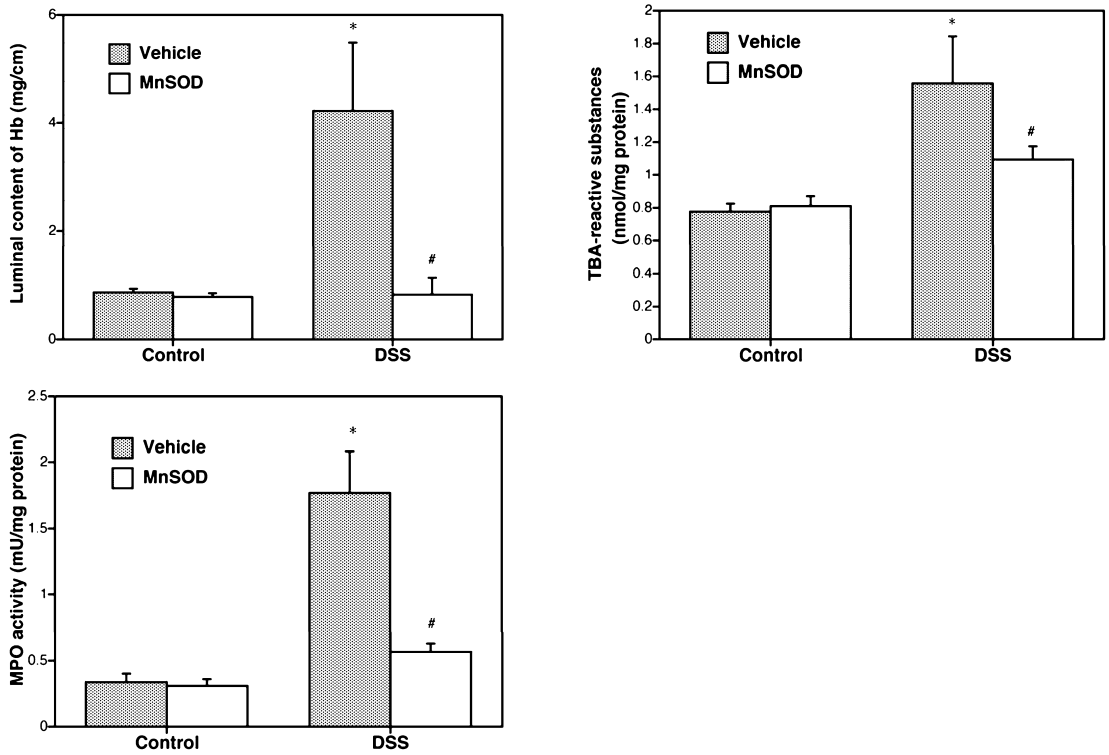

Fig. 3. Effects of Mn-SOD on DSS-induced colitis in mice. Acute colitis was induced by DSS administered at 8% w/v in the drinking water in BALB/c female mice. MnSOD (50,000 U/kg) was dissolved in physiological saline, and administered by intraperitoneal injection for 7 days. Reprinted with permission [*6*].

that superoxide is implicated in the regulation of the endothelial cell adhesion molecule expression and the subsequent initiation of leukocyte-endothelial cell adhesion in different experimental models of inflammation.

Recently, Morohoshi *et al.* [*16*] investigated the contribution of neutrophil elastase in a murine acute colitis model. They have shown that the neutrophil elastase enzyme activity is significantly elevated in both the plasma and colonic mucosal tissue in UC patients compared with healthy controls, and that ONO-5046, a neutrophil elastase specific inhibitor exerts therapeutic effects in DSS-treated mice by significantly reducing weight loss and histological score. In addition to its protease activity contributing to tissue destruction, it has been reported that neutrophil elastase enhances the migration and adhesion of neutrophils [*18*]. ONO-5046 has already been clinically used for the treatment of acute respiratory distress syndrome in Japan, and no serious adverse effects have been reported. Therefore, ONO-5046 might actually have the potential to be a new therapeutic approach for patients with UC.

Neutrophil Activation in Ulcerative Colitis

In patients with UC, the circulating levels of neutrophils are found to be up to three times higher than the levels in healthy controls [*19*]. Morphological and functional evidences of activation of circulating neutrophils have been reported in patients with inflammatory bowel disease. In 1991, McCarthy *et al.* [*20*] found the increase in the number of polarized neutrophils, determined by quantitative light microscope examination, in patients with active stage of UC compared to those with quiescent colitis or normal subjects. Functionally, neutrophils obtained from UC patients have a significantly higher response than those from controls following phorbol myristate acetate, formyl-methionyl-leucyl-phenylalanine, and zymosan administration [*21*].

Anezaki *et al.* [*22*] have investigated the correlation between IL-8, and MPO or luminal-dependent chemiluminescence in inflamed mucosa of UC. They have found that luminol-dependent chemiluminescence of biopsy specimens in active UC is markedly increased compared to those in inactive UC and controls (Fig. 4), and that the levels of IL-8 are closely correlated to the intensity of chemiluminescence or MPO levels. In UC, IL-8 mRNA was found mainly in macrophages, and also in neutrophils and colonic epithelial cells [*23*]. In addition, increased production of IL-8 peptides and expression of IL-8 mRNA is observed in the inflamed mucosa of patients with UC [*24*]. Since IL-8 is not only a chemoattractive substance but also a neutrophil-activating substance to release oxygen radicals from neutrophils, the data by Anezaki *et al.* suggest that most of the infiltrating neutrophils in the colonic mucosa of UC is activated.

Human neutrophil lipocalin (HNL) may be a more sensitive marker for neutrophil activation in the colonic mucosa

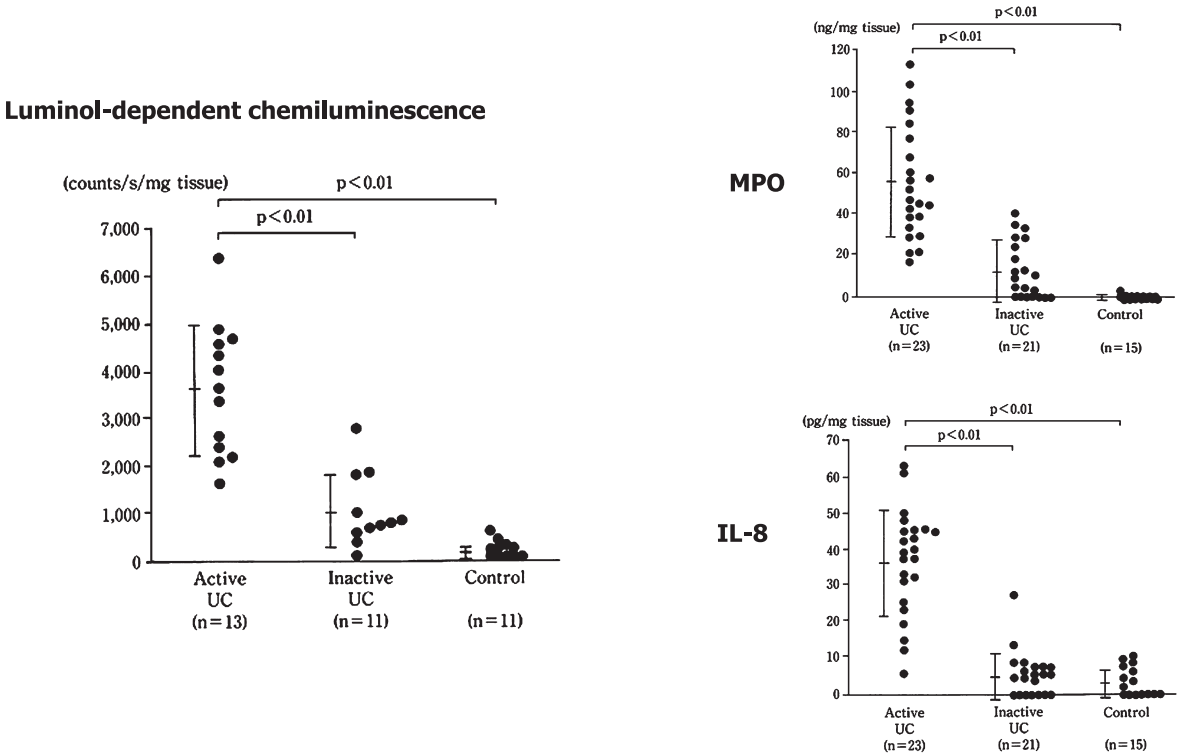

Fig. 4. Luminol-dependent chemiluminescence in patients with ulcerative colitis. Reprinted with permission [*22*].

compared to MPO, because primary granules including MPO are not unique to the neutrophil granulocyte. HNL is a secondary granule protein unique to the neutrophil. Carlson *et al.* [*25*] have demonstrated that HNL levels in colorectal perfusion fluids increase in ulcerative colitis and proctitis, and suggest that HNL may serve as a specific marker of intestinal neutrophil activation in UC.

Leukocytapheresis Therapy in Ulcerative Colitis

Leukocytapheresis procedure

The Adacolumn is an example of a medical device that can selectively remove activated granulocytes and monocytes/macrophages together with small populations of lymphocytes (Fig. 5). The Adacolumn is a single use adsorptive type apheresis column, with a volume of 335 ml, filled with 220 g of cellulose acetate beads of 2 mm diameter as the column adsorptive carriers. Leukocytapheresis using the Adacolumn is performed in out-patient clinic for patients with mild to moderate symptoms, and patients with severe symptoms were hospitalized for apheresis. The process of performing leukocytapheresis is relatively simple. Prior to apheresis, the system is primed by saline-containing anticoagulant, nafamostat mesilate or heparin, and during apheresis saline containing those anticoagulants is continuously administered into the column. The commonly used apheresis time and blood flow rate are 60 min and 30 ml/min, respectively. During these procedures, the carriers absorb about 65% of granulocytes, 55% of monocytes and 2% of lymphocytes from the blood in the column. These are the leukocytes that bear the so-called FcgR and complement receptors. One apheresis session per week for five consecutive weeks has been a standard protocol. Recently, other protocols with two sessions per week or a total of 10 apheresis sessions have been used for patients with severe UC.

Anti-inflammatory/immunomodulatory actions of the Adacolumn

Although the aim of treatment with Adacolumn has been to remove excess and activated granulocytes and monocytes from the circulation, recent reports have demonstrated the anti-inflammatory and immunomodulatory actions of this leukocytaheresis. At first, during passage of blood through the Adacolumn, most of the activated leukocytes adhere to the cellulose acetate beads. In the intra-column response, the adhered leukocytes release an array of anti-inflammatory substances. It has been found the increase in interleukin-10 [*26*], soluble tumor necrosis factor (TNF)-α receptor I and II [*27*], interleukin-1 receptor antagonist, and hepatocyte growth factor (HGF) [*28*]. Soluble TNF-α receptors are reported to neutralize TNF without involving TNF-like actions. IL-1ra has an essential role in the control of inflammation in the intestinal mucosa, while HGF is known to promote mucosal epithelial cell regeneration. Secondary,

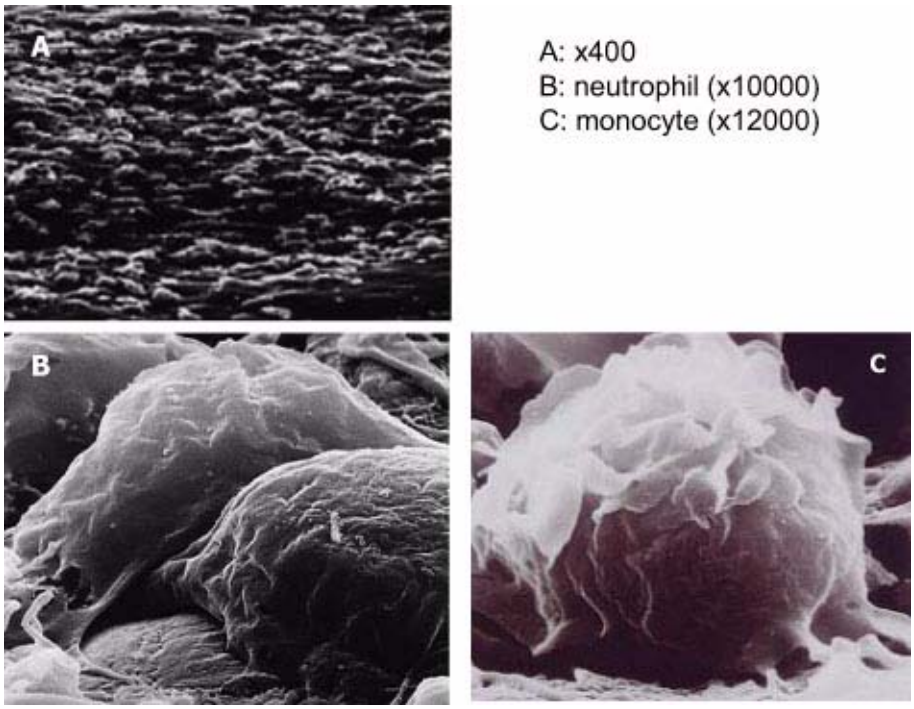

Fig. 5. Electron micrograph showing leukocytes, mostly neutrophils and monocytes adsorbed onto an Adacolumn cellulose acetate carrier. Photograph by Dr. A. Saniabadi of Japan Immunoresearch Laboratories.

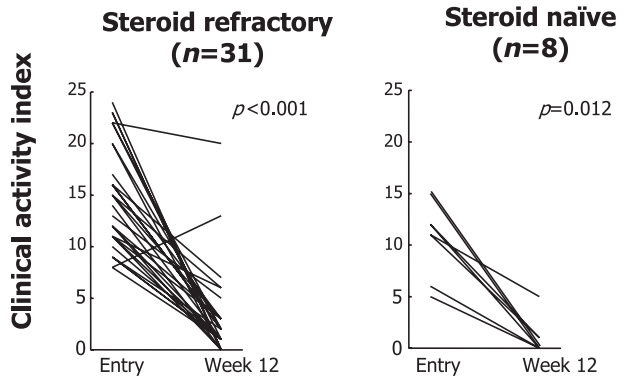

Fig. 6. Granulocyte adsorptive apheresis therapy for steroid-refractory or -naïve ulcerative colitis. Reprinted with permission [*32*].

the expression of both L-selectin and the chemokine receptor CXCR3 were dramatically reduced and were sustained well beyond the last leukocytapheresis session [*29*, *30*], whereas the expression of the leukocyte integrin Mac-1 (CD11b/CD18) was upregulated. Finally, a study by Kashiwagi *et al.* [*30*] shows that the proportion of naïve or immature neutrophils ($CD10^-$ neutrophils) in the circulation significantly increases during Adacolumn leukocytapheresis, indicating that immature granulocytes had originated from the bone marrow.

Indications for leukocytapheresis

According to the Guideline of the Investigation and Research Committee of Inflammatory Bowel Disease of the Ministry of Health and Welfare of Japan, leukocytapheresis has been mainly used for patients with steroid-refractory and steroid-dependent moderate-to-severe UC. Recently, steroid-naïve patients with mild UC were also treated with leukocyte apheresis.

Clinical efficacy

Recently, Yamamoto *et al.* summarized the safety and clinical efficacy of leukocytapheresis using the Adacolumn (granulocyte and monocyte adsorptive apheresis: GMCAP) for ulcerative colitis. The efficacy of GMCAP was evaluated using clinical activity index or disease activity index scores [*31*]. In a prospective multicenter trial in Japan, 53 patients refractory to conventional drug therapy were treated with the standard GMCAP protocol (five apheresis sessions for five consecutive weeks) in combination with prednisolone. After the treatment, 21% and 37% of patients achieved remission and improvement, respectively, and the mean daily dose of prednisolone per patients was reduced from 24.4 mg at enrollment to 14.2 mg after GMCAP therapy. In addition to steroid-refractory UC patients, it has been shown that 88% of steroid-naive patients achieved remission by GMCAP therapy [*32*]. In summary, clinical data obtained in Japan suggest that leukocytapheresis is an effective adjunct to therapy for UC to promote remission, taper conventional drug dosage, and potentially reduce the number of patients who require colectomy. The results should further understanding of the pathophysiology of inflammatory bowel disease.

Conclusions

We have summarized the recent advances in the role of neutrophils-dependent oxidative stress in intestinal inflammation of UC. Although basic research has demonstrated that several antioxidants and inhibitors of neutrophils-derived enzymes inhibit the inflammatory responses in intestinal tracts, further studies will be needed to clarify the precise mechanism of these agents. In clinically, the successful outcome of leukocytapheresis for UC clearly confirms the crucial role of neutrophils in the pathogenesis of this disease.

Abbreviations

DSS, dextran sodium sulfate; GMCAP, granulocyte and monocyte adsorptive apheresis; HGF, hepatocyte growth factor; HNE, 4-hydroxy-2-nonenal; HNL, human neutrophils lipocalin; ICAM-1, intercellular adhesion molecule 1; IL-8, interleukin-8; MPO, myeloperoxidase; PAMPs, pathogen-associated molecular patterns; PECAM-1, platelet endothelial adhesion molecule-1; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, thiobarbituric acid; TNBS, trinitrobenzene sulfonic acid; TNF, tumor necrosis factor; TLR, Toll-like receptor; UC, ulcerative colitis; VCAM-1, vascular cell adhesion molecule 1.

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